Giving eyespots a shiner: Pharmacologic manipulation of the Io moth wing pattern [version 1; referees: 2 approved with reservations]

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Abstract
Our knowledge of wing pattern formation in Lepidoptera has advanced significantly in recent years due to the careful examination of several groups of butterflies. The eyespot is a prominent feature of Lepidoptera wing pattern, especially in the family Saturniidae. The present study examined how sulfated polysaccharides, which are known to simulate cold shock effect in nymphalid butterflies, affected the wing pattern formation of the Io moth, *Automeris io* (Saturniidae). Prepupae and pupae of this species were subjected to injections of heparin and cold shock. While the cold shock had little to no effect on wing pattern, the aberrations resulting from heparin injections consisted of moderate to profound increases in melanism around the eyespots. The resulting aberrations are dubbed ‘Black Eye’ and ‘Comet Eye.’ Most other known aberrations of *Automeris io* eyespots are summarized, illustrated and named.

Open Peer Review

Referee Status: ? ?

Invited Referees
1
2

version 1
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1 Arnaud Martin, The George Washington University, USA
2 Jeffrey M. Marcus, University of Manitoba, Canada

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Author roles: Sourakov A: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Resources, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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Introduction
While our understanding of the mechanisms involved in butterfly wing pattern development has been increasing exponentially in the recent two decades, the work has been largely limited to butterflies such as Junonia, Heliconius, Papilio and Bicyclus. Thanks to these ‘model’ genera, we now understand homologies among wing pattern elements and the adaptive radiation that led to the kaleidoscope of intriguing ‘designs’ found among ca. 160,000 Lepidoptera species (Martin & Reed, 2010).

Natural and artificially generated aberrations serve as windows into the developmental mechanisms and evolutionary history of animals. In addition to many naturally occurring melanic aberrations and some melanic recessive phenotypes that can be obtained and/or maintained through inbreeding, the dark markings of Lepidoptera wings can sometimes be amplified by the timely application of a colder regime to the immature stages (e.g., Sourakov, 2015 and references within). Serfás & Caroll (2005) first demonstrated that injections of heparin into the early pupal stage can simulate cold shock and alter wing patterns in similar ways. Martin & Reed (2014) utilized heparin injections to understand genetic controls and homologies among separate wing pattern elements.

Eyespots are characteristic of many Lepidoptera, and considerable advances have been made towards understanding their evolution (Monteiro et al., 2006). In Automeris io, a species whose name, if anglicized (‘Eye’Oh!’), invokes associations with its pair of magnificent dorsal hindwing eyespots that are exposed when the moth (otherwise cryptic) is threatened. Several recessive mutations causing deformations of the black ring surrounding the dark blue eyespot with a white center have been obtained through mutations causing deformations of the black ring surrounding the dark blue eyespot with a white center have been obtained through and maintained through inbreeding, the dark markings of Lepidoptera. In addition to many naturally occurring melanic aberrations and some melanic recessive phenotypes that can be obtained through inbreeding, the dark markings of Lepidoptera wings can sometimes be amplified by the timely application of a colder regime to the immature stages (e.g., Sourakov, 2015 and references within). Serfás & Caroll (2005) first demonstrated that injections of heparin into the early pupal stage can simulate cold shock and alter wing patterns in similar ways. Martin & Reed (2014) utilized heparin injections to understand genetic controls and homologies among separate wing pattern elements.

Table 1. Aberrations of dorsal hindwing eyespots found in Automeris io.

<table>
<thead>
<tr>
<th>Aberration name</th>
<th>Description</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Black eye”</td>
<td>Figure 1A – area between eyespot and outer black ring entirely black</td>
<td>A. Sourakov</td>
</tr>
<tr>
<td>“Broken eye”</td>
<td>Figures 3B, C – vertical streaks of black medially of the eyespot</td>
<td>T.R. Manley</td>
</tr>
<tr>
<td>“Teardrop”</td>
<td>Figure 3D – eyespot shape modified, with an appendix extending towards wing base</td>
<td>T.R. Manley</td>
</tr>
<tr>
<td>“Caecus”</td>
<td>Figure 3A – eyespot disappears, masked by black pigment</td>
<td>wild</td>
</tr>
<tr>
<td>“Comet eye”</td>
<td>Figure 2A – black ring around eyespot with smudges extending towards wing base</td>
<td>A. Sourakov</td>
</tr>
<tr>
<td>“Barley eye”</td>
<td>Figure 3F – black ring uneven, bulging or protruding locally</td>
<td>A. Sourakov</td>
</tr>
<tr>
<td>“Winking eye”</td>
<td>Figure 3E – blue circles forming eyespots are of uneven size on left and right wings</td>
<td>A. Sourakov</td>
</tr>
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</table>

In the present study, an attempt has been made to replicate these aberrations with heparin injections to the prepupal and pupal stages, as well as by cold shock. The results of the former manipulations, while not replicas of previously known aberrations, are quite dramatic and are illustrated here along with some other aberrations, both known and those previously unrecorded.

Methods
Representatives of five broods of Automeris io from local stock (over 300 caterpillars) were reared on sugarberry under ambient condition in Gainesville, Florida, in the fall of 2016 resulting in 130 pupae. While the caterpillars were pupating in November, ten randomly selected pupae were injected, using a 10µl syringe, under a wing with ~5mg (10µl (1 drop) of heparin dissolved in distilled water). Additionally, one pupa was injected a day before pupation with half of that amount, and a dozen were subjected to cold shock in the refrigerator (4–5 C°) overnight. Ten other pupae were injected with 10µl of mannitol (saturated solution), and the rest were left untreated. All pupae were kept under ambient conditions during diapause, until they began emerging in May 2017.

Results and discussion
While most of the pupae that were injected did not emerge, one female with a strongly modified wing pattern emerged from a pupa injected with 5mg of heparin (Figure 1A.i). Injection must have damaged the right hindwing, so it did not spread properly (Figure 1A.ii), but the left side was structurally intact.

![Figure 1. A. “Black eye” aberration. A female of the Io Moth, Automeris io, with wing pattern altered by injection 5mg of heparin (sulfated polysaccharide) into the early pupal stage. Voucher FLMNH-MGCL#289216. B. A normal A. io female from the same brood. (i) dorsal, (ii) ventral surface. Photos by Andrei Sourakov.](image-url)
control sibling female is illustrated in Figures 1B.i and B.ii for comparison.

A less aberrant male, whose prepupa was injected with 5mg of heparin a day before pupation, also emerged (Figures 2A.i, 2A.ii), different in its dorsal hindwing eyespots from all control siblings (e. g., Figure 2B). The cold-shocked and the mannitol-injected individuals showed no obvious deviation from expected wing patterns. Most of the cold-shocked and the mannitol-injected individuals showed no obvious deviation from expected wing patterns. The slight wing pattern changes (Figures 2C and 2D) exhibited by two females, cold-shocked as prepupae, suggest that cold shock may have some melanization-inducing effect and perhaps, if administered differently, may potentially have a greater effect on the phenotype.

On the dorsal hindwing of aberrant female (Figure 1A.i), the blue eyespot center is smaller than that of the control siblings (e.g., Figure 1B.i), due to the infraction of melanin from the surrounding black ring. Heparin injection must have enhanced or prolonged the process of expansion of black pigment once it formed in the black ring around the blue scales. Judging by the wild aberrant male, such melanization process can go as far as eliminating the eyespot entirely (Figure 3A). In the heparin-injected aberrant female, the process of expansion mostly occurred

Figure 2. A. “Comet eye” aberration. A male of the Io Moth, Automeris io, from prepupa injected with 2.5mg of heparin a day prior to pupation. FLMNH-MGCL#289217. B. A normal A. io male from the same brood. Voucher FLMNH-MGCL#289218. C, D. Slight aberrations (“Comet eye” and “Barley eye”) of the black ring around eyespots in two A. io females cold-shocked as prepupae. Photos by Andrei Sourakov.

outwards, so that the black ring around the eyespot merged with the normally thin black EIII line of the hindwing margin, which too had widened (Figure 1A.i).

A name “Black Eye” is proposed for the aberration shown in Figure 1A, following the tradition started by Manley (1978, 1990), who gave genetic aberrations of Automeris io names, such as “Broken eye,” (Figures 3B and 3C) and “Teardrop” (Figure 3D). Names of all aberrations, old and newly proposed, are summarized in Table 1.

As can be observed by comparing the “Black Eye” to its sibling (Figure 1B.i), the DI element of the forewing also underwent modification, as if it were smudged from its center along the veins toward the distal part of the wing. The ventral surface of the wing in “Black Eye” shows considerable expansion and diffusion of the small and compact black ring of control specimens around the small white ventral eyespot (Figures 1A.ii and 1B.ii).

Scholars engaged in wing pattern research have recently identified 27 genes associated with wing melanin production (Zhang et al., 2017). It is hoped that the present publication, while documenting unique aberrations in a single species, will be useful in the future work directed at understanding wing pattern evolution and development in general.

Competing interests
No competing interests were disclosed.

Grant information
The author(s) declared that no grants were involved in supporting this work.

Acknowledgements
I thank Larry Gall, the Entomology Collection Manager of the Peabody Museum at Yale University, for granting access to the collection and for permission to photograph and publish Automeris io aberrations reared by late T. R. Manley. I am also thankful to my family for their tolerance during the Thanksgiving break, when I was occupied with this experiment, and especially to my daughter Allie for proofreading this manuscript.

References

Reference Source
Published Full Text
Reference Source
Open Peer Review

Current Referee Status: ??

Version 1

Referee Report 17 August 2017
doi:10.5256/f1000research.13271.r24774

Jeffrey M. Marcus
Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada

This manuscript describes a very promising approach to expanding the experimental study of color pattern development beyond the select group of model butterfly species that have received the bulk of the attention thus far. The Saturniid moth Automeris io was used for these experimental studies. Also described are A. io specimens derived from breeding experiments and wild-caught specimens with aberrant phenotypes.

While I enjoyed reading the manuscript, there are a number of changes that I would like to suggest to the author:

Clarification of methods:
There are a number of methodological details that should be clarified.
1. “sugarberry”. Please give scientific name. Is this Celtis laevigata?
2. Staging of pupae. As much detail as possible should be given here about when and how the injections were done relative to the pupal molt. How many hours at what temperature? Was the cuticle sclerotized yet? Where on the experimental specimens’ bodies did the injections take place? Under what conditions were the animals allowed to recover from their injections? What was the manufacturer and purity of the heparin used? For coldshock, how many hours is “overnight”?
3. Control injections: what was the rationale for using manitol? Many experiments pair heparin with chondroitin sulfate B (keratan sulfate) as a negative control because it is structurally similar to heparin, but lacks the biological activity associated with heparin.
4. Overwintering conditions: the author reports that the experimental animals were kept at ambient conditions until emergence the following spring. Were they kept indoors or outdoors? If indoors what was the typical temperature and lighting conditions during this period? If outdoors, try to provide descriptors of climatic conditions during the appropriate period using National Weather Service or other data.

Clarification of results:
Additional clarification of the results is also required.
1. The author reports that most of the injected individuals did not emerge. How many failed to emerge? Were there any differences in eclosion rate between the heparin injected individuals and the chondroitin sulfate B injected individuals?

2. What was the emergence rate of unmanipulated individuals? Was it different from the emergence rate of cold-shocked individuals?

Reinterpretation of Results and Elaboration of Discussion:

1. The author should keep in mind that Lepidopteran color patterns can be altered by manipulation of the developmental processes responsible for determining color patterns as well as by manipulation of the developmental processes responsible for color pattern differentiation. Determination processes might involve cell-cell signaling by signal transduction processes, while differentiation of color patterns involves the expression and regulation of biosynthetic pathways responsible for pigment synthesis. Manipulations such as cold-shock might have effects on both kinds of developmental processes, if they are taking place at the time of the manipulation. Additional references to prior work that examines the effects of coldshock (Nijhout 1984; Serfas and Carroll 2005; Mahdi et al. 2010; Dhungel and Otaki 2013) might be warranted.

2. Everything that we know about the action of heparin suggests that it interacts with signal transduction pathways such as wingless/wnt (Binari et al. 1997), and there is no evidence that it interacts with melanin pigment biosynthesis. Discussing the experimental results of heparin injection from this study in reference to what is known about wingless/wnt signaling and its effects on color pattern determination in insects (Carroll et al. 1994; Monteiro et al. 2006; Martin and Reed 2010; Werner et al. 2010) would be highly desirable. I also urge the author to extend the discussion further and to connect his work with prior work on Saturniid moth color pattern development including both classic cautery studies (Henke 1933; Henke 1944) and studies of gene expression (Monteiro et al. 2006). Interestingly, the discal eyespots of Automeris io are positioned on top of crossveins, structures that also employ wingless/wnt signaling during their development (Conley et al. 1997; Marcus 2001). Relating eyespot development to underlying crossvein development in Saturniid moths, perhaps through modulation of the wingless/wnt pathway would be a very interesting research trajectory to pursue. Understanding how discal eyespots in some Saturniid moths are both similar to and different from border eyespots in butterflies would be a very interesting avenue for further studies of the evolution and development of Lepidopteran color patterns.

I would like to encourage the author to consider these points in his revisions and also to continue his experimental explorations in future work.

References


Reference Source


**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

No

**If applicable, is the statistical analysis and its interpretation appropriate?**

Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
Referee Report 04 August 2017

doi:10.5256/f1000research.13271.r24773

Arnaud Martin
Department of Biological Sciences, The George Washington University, Washington, DC, USA

This manuscript presents some exciting pattern aberrations observed in the eyespots of the Io Moth, including specimens obtained from drug injections, a wild-caught individual, and previously undescribed collection specimens.

I enjoyed reading the manuscript and thank the author for publishing this. The reported phenotypes provide valuable information on the formation of eyespot rings in this particularly spectacular species.

I noticed the following minor issues.

- What was the rationale for injecting mannitol?
- Serfas and Caroll (2005) -> Carroll with two r (typo)
- Methods: "Ten randomly selected pupae were injected"
  Please provide more details if possible on the staging. Were they tender and relatively fresh? Is there a way to determine the maximum age they were at, or a time estimate based on their cocoon spinning?
  5mg of heparin is a large dose, so it may have killed the younger pupae, while the aberrant female came out by chance by being at a more resilient stage (or was accidentally injected with much less compound)
- Methods, please confirm the injected concentration of the injected heparin (apparently 0.5mg/uL), and provide the exact origin of the compounds (sodium salt? molecular weight? manufacturer?)
- Figure 1 Legend: the abbreviation for DI BR and EIII are missing.
- The author is wrong calling the outer black line "EIII". Schwanwitsch 1956 and Henke 1936 (in 3 other Saturniids) suggest this is M1 (central symmetry system outer band). I am personally inclined to say that while it may not be M1 in A. io ... it is certainly not EIII
- "A less aberrant male, whose prepupa was injected with 5mg of heparin a day before pupation, also emerged"
  I believe the author meant "A less aberrant male, whose prepupa was injected with 2.5mg of heparin ONE day before pupation, also emerged"
- "Heparin injection must have enhanced or prolonged the process of expansion of black pigment once it formed in the black ring around the blue scales."

There is a way to deepen the discussion here, and I will try to explain briefly (feel free to use this suggestion).

The A. io Discal Spots (DI = Discalis I element of the Schwanwitsch pattern homology system) are stereotypical patterns that are always overlapping with the discal crossvein. Martin and Reed (2010) suggests that these spots always express the wingless morphogen in Lepidoptera, and Martin and Reed...
suggests that DI elements are responsive to heparin treatment in nymphalids. Interestingly, heparin is known to enhance wingless signaling in Drosophila: Baeg et al. (2001), Binari et al (1997) and Greco et al (2001).

Thus, the eyespot ring expansions observed upon heparin injection here suggest the exciting possibility that wingless, or perhaps other heparin-sensitive morphogens, are deployed during pre-pupal and pupal development to pattern different aspects of the discal ocelli structure.

To be honest, the current insight about melanization is a little too phenomenological, because melanin pigment synthesis happens much later in development and there is no known interaction between heparin and melanin biosynthesis...

References

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others? Partly

If applicable, is the statistical analysis and its interpretation appropriate? Not applicable

Are all the source data underlying the results available to ensure full reproducibility? Yes

Are the conclusions drawn adequately supported by the results? Yes

Competing Interests: No competing interests were disclosed.
**Referee Expertise**: Developmental Genetics, Lepidoptera

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.