Scientific Note: The Emperor's new clothes: radical transformation of the wing pattern in *Asterocampa clyton* caused by heparin

Andrei Sourakov

McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

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Abstract: Sulfated polysaccharide injection at the early pupal stage caused a radical transformation in the wing pattern of the Tawny Emperor butterfly, *Asterocampa clyton*. This butterfly is a member of the family Nymphalidae, which has been at the forefront of the latest research into wing pattern genetics. Induced wing pattern changes are likely a result of changes in the activity of the *Wnt* cell-signaling pathway, which is presumably deployed when the timely injection of the pharmacological product heparin were made into the wing area of the developing pupa approximately five hours after pupation. These changes, which are consistent with previous experiments by others on other nymphalid species, had a profound effect on the overall appearance of the insect. Should individuals like the one produced by this experiment be encountered in the wild, it would undoubtedly be mistaken for a new, undescribed species, as it bears little superficial resemblance to its siblings.

INTRODUCTION

Heparin injection at the early pupal stage caused a radical transformation in the wing pattern of the Tawny Emperor butterfly, *Asterocampa clyton* (Fig. 1A, B). This butterfly is a member of the family Nymphalidae, which has been at the forefront of the recent research into wing pattern genetics, with butterfly patterns being manipulated with stunning results. For example, changes induced by *optix* gene knockout via CRISPR deleted color orange in the Gulf Fritillary, *Agraulis vanilla*, and induced iridescence in the Buckeye, *Junonia coenia*, (Zhang *et al*., 2017). The activity of patterning molecules of the *Wnt* pathway can be altered by the timely injection of the pharmacological product heparin (e.g., Serfas and Carroll, 2005, Martin & Reed, 2014), which can lead to a complete overwriting of the normal pattern, or expand some of the pattern elements, depending on the stage of development and the dose.

Based on experiments on *Euphydryas chalcedona*, *Vanessa cardui*, and *Agraulis vanillae* (Martin & Reed, 2014), nymphalids express several *Wnt* genes. The heparin treatment provides an experimental way to disrupt the patterning system controlled by *Wnt* genes, and, within narrow limits in which the insect can survive the heparin injection, the higher dose leads to bigger change (e.g., Serfas & Carroll, 2005 (Fig. 1); Martin & Reed, 2014 (Figs. 5,6); Sourakov, 2017 (Fig. 2)).

While the current note shows only the preliminary results of exploring *Wnt*'s roles in yet another nymphalid genus, *Asterocampa*, it may provide research teams engaged in wing pattern studies with an incentive to use this easy-to-rear, common, and attractive species as yet another subject for wing pattern genetics research.

MATERIALS AND METHODS

A group of gregarious caterpillars of the Tawny Emperor butterfly, *Asterocampa clyton*, was found in Gainesville, Florida, USA on 14 October, 2017, on the sugarberry (or hackberry) tree, *Celtis laevigata*. They were reared to pupation on this host plant. Where pupation was observed, pupae were injected within 12 hour period superficially through the membrane separating A3 and the forewing. Heparin sodium salt, Porcine, manufactured by MP Biomedicals, Inc., was dissolved at 1 part salt/2 parts distilled water by weight. The injection was made on one side using a 0.3ml hypodermic syringe with a drop (ca. 3-5µl). Variation between injected volumes with the hypodermic needle technique and within timing after pupation may explain the heterogeneity in survival, so for reproduction of the results, it is recommended to inject more diluted versions and at a range of volumes and to monitor the pupation time more closely. Twenty seven control (untreated) pupae emerged as normal adults as in Fig. 1A, while all experimental pupae (10) either underwent transformation or died. All adults that emerged did so in November-December 2017. The specimen featured in Fig. 1B resulted from injecting an approximately 5-hour-old pupa (Fig. 2) and was the only one of the experimental batch that emerged and spread its wings, while other transformed individuals were assessed by dissecting dead pupae under the microscope. The spread experimental specimen is deposited in the collection of the McGuire Center for Lepidoptera, Florida Museum of Natural History, University of Florida, MGCL voucher #291689, along with several control specimens (#291684-291688, 291931-291937).

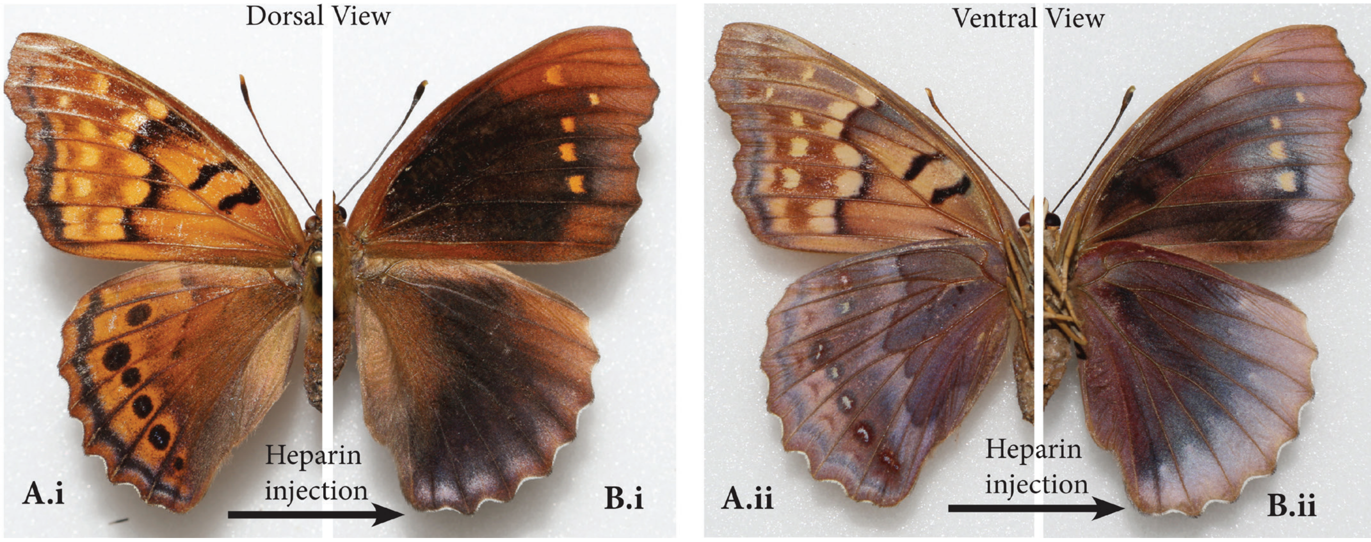

Fig. 1A,B. The Tawny Emperor, *Asterocampa clyton*: A. normal; B. injected with sulfated polysaccharide (heparin) at the early pupal stage. (i) Dorsal side, (ii) ventral side.

RESULTS AND DISCUSSION

Heparin injection caused a radical transformation of the wing patterns in the Tawny Emperor butterfly, *Asterocampa clyton* (Fig. 1A, B), demonstrating that all wing surfaces of this nymphalid butterfly may be drastically changed by heparin. The emergence of 27 sibling pupae that were not manipulated as normal adults leaves little doubt that the heparin was responsible for the transformation of the specimen in Fig. 1B, which was injected within 9 hours after pupation The transformation is also consistent with results obtained by others (e.g., Martin & Reed, 2014).

Such dramatic changes in the wing pattern of *Asterocampa* could have been anticipated when one considers the diversity of roles the *Wnt*-family secreted ligand *WntA* plays in nymphalids such as *Heliconius*, *Agraulis*, *Vanessa*, *Pararge* and *Danaus* (Mazo-Vargas *et al*., 2017). The previous effect of large doses of heparin on expansion of, for example, dorsal black spots on the dorsal side of *Euphydryas* and *Agraulis* has been demonstrated by Martin & Reed (2014). In yet another nymphalid, the White Admiral, *Limenitis arthemis arthemis*, heparin eliminated the characteristic white bands of this morph (Gallant *et al*., 2014). As was shown in *Limenitis* and *Heliconius*, *Wnt* signaling delimits melanic territories, and the expression of *WntA* outlines the boundaries of wing pattern elements, such as bands and spots (Martin *et al*., 2012; Gallant *et al*., 2014, reviewed in Martin & Courtier-Orgogozo, 2017). The latter review (p.75) suggests that "with a total of 18 alleles in 7 species, all associated with wing color pattern variation, *WntA* can be seen as a genuine genetic hotspot of adaptation..." There is a certain level of polymorphism in brightness, extent and contrast of the dark markings on *Asterocampa clyton*'s wings (Warren *et al.*, 2013), and, as suggested by Martin & Courtier-Orgogozo (2017, p. 79), such polymorphism may "map to a *Wnt*-pathway gene or to a gene that can modify the output of the *Wnt* signaling pathway."

Fig. 2. Pupa of *Asterocampa clyton* shown in Fig. 1B after injection. The pupae of this species are soft and translucent upon pupation, but harden and lose translucency within the first 24 hours.

CONCLUSIONS

Judging by the fact that most of the injected pupae died, and by the fact that the supposedly delivered heparin dose is much higher than heretofore was used successfully by other reserachers, the dose must have been close to the maximum an insect could bear. It is therefore suspected that the observed is the maximum degree of change that *Asterocampa* wing pattern can undergo by tweaking the expression of the *Wnt* regulatory pathway. The regulator(s) affected by heparin, resulted in a dramatic black pattern expansions on the dorsal side, and diffusion of the pattern elements on the ventral side. Several wing pattern elements remained unaffected and must be not under *Wnt* control. The treatment provided this "Emperor" with entirely "new clothes," pointing out similarities and differences in genetic wing pattern control with other nymphalid butterflies on which similar experiments have been conducted.

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