Painting Wings - Evidence for a Role of Ca^{2+} in Butterfly Wing Phenotypic Plasticity

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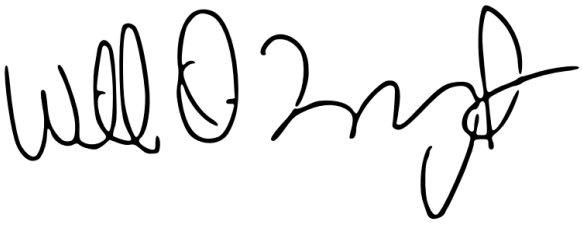

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Abstract

Wing patterns in butterflies serve several functions such as mimicry, sexual dimorphism, camouflage, and temperature regulation. Based on temperature adaptations by butterflies involving change in brightness and calcium mediated temperature responses. Here, we investigate the role of calcium in mediating the expression of light and dark scales. Through utilization of the butterfly *Vanessa cardui* as a model, I investigated how fluctuations in cytosolic calcium levels can potentially influence the development of wing brightness. Specimen brightness was manipulated with tungstate and thapsigargin injections. Tungstate has been shown to modify butterfly wing patterns but its mechanism of action has yet to be identified. Thapsigargin is a well-studied drug known to result in increases of cytosolic calcium similar to that of natural heat responses. Through utilization of these compounds, my hope was to reach a better understanding of the specific ways in which wing patterns are regulated by calcium. The specimens manipulated with pharmacological injections of thapsigargin showed an increase in the number of light scales within the injected area. Alternatively, specimens injected with tungstate showed a larger proportion of dark scales. Overall, findings suggest that calcium potentially plays a significant role in organizing wing pattern development.

Introduction

Biological Significance: Butterfly wing pattern plasticity is critical for survival. Butterfly wing patterns serve various important functions, such as camouflage, sexual dimorphism, and mimicry. An important way that wing patterns support survival is by adapting to environmental conditions such as temperature. Butterflies often exhibit different phenotypes depending on the climate of their habitat. For example, palearctic butterflies in higher altitudes with colder climates have darker wings than in lower altitudes (Cassel et al., 2020). Darker wings absorb more solar radiation. Adaptations like this translate to longer flight time, more egg laying and greater reproductive success (Guppy, 1986).

Figure 1. *Junonia ceonia* seasonal forms. Six forms of *Junonia evarete* shown. Having a seasonal form allows the species to maximize flight time and egg laying in each season (Jameson, 2017). Generally, different seasonal cues like temperatures impact the butterfly during its larval and pupal development. In the case of cooler fall temperatures, it results in darker forms, smaller eyespots and cryptic fallen leaves wing coloration [wing 2-3]. Summer forms are lighter in color with larger eyespots [wing 1]. There are also intermediate forms that occur in some populations [wing 4-6]. Forms can also be habitat and host plant specific (Dennis, 1989). Once developed within the pupa, adult butterflies cannot alter their wings.

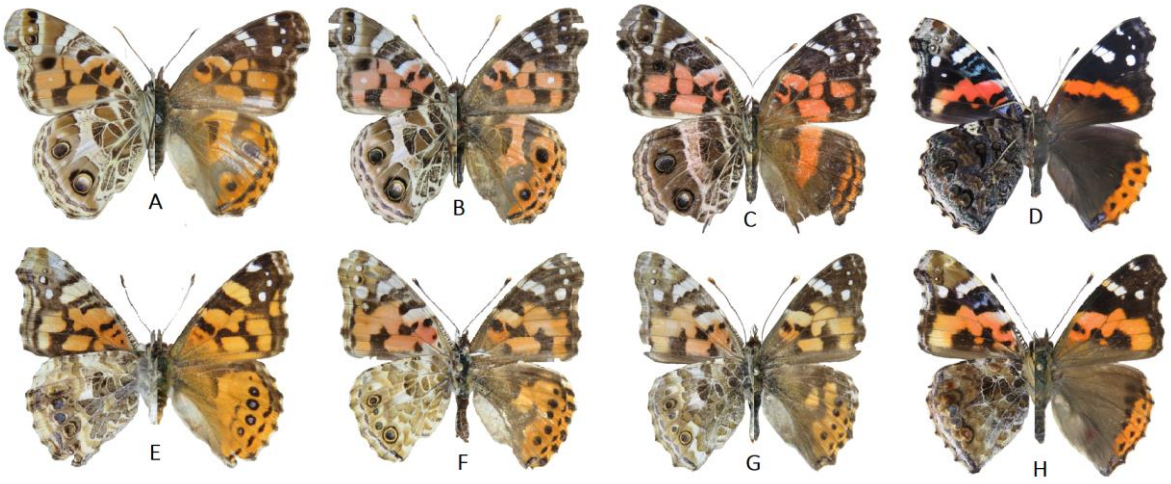


Figure 2. *Vanessa* species examples. *V. brasiliensis* [Argentina] (A), *V. brasiliensis* [Tiradentes, Brazil] (B), *V. brasiliensis* [Tiradentes, Brazil] (C), *V. atalanta* [Bloomington, USA] (D), *V. anabella* [San Diego, USA] (E), *V. cardui* H) *V. indica* [Chongqing, China] (F & G).

Literature Review: Interest in wing pattern development first originated in the late 1700s when English butterfly collectors began describing variations in butterfly wing patterns as forms. Subsequently in the 1850s there were observations associating temperature with butterfly wing forms and in 1890 cold and heat shock experiments began. In 1897 a seasonal dimorphism hypothesis was published after two butterfly species were found to be 2 different forms of the same species (Mayer, 1897). In 1998, a hormone or oxyanion influencing phenotype change in response to temperature was found to be within the hemolymph (Otaki, 1998). In 2015, the presence of spontaneous long-range calcium waves was identified during pupal development (Ohno et al., 2015). In 2017 two genes, WntA and Optix were found to be involved in regulating patterns and structural iridescence in butterflies (Zhang et al. 2017, Mazo-Vargas et al. 2017). In the same year, calcium signaling was identified in eyespots of butterflies and found to be an early trigger in eyespot development (Ozsu et al., 2017).

Physiological Mechanisms: What mediates wing pattern development? The colors of butterfly wings are composed of pigments (ommochromes and melanins) and microstructures that reflect light. Structures are mainly localized on the surface of the wing scales and are important for contributing to color and are strictly responsible for blue and similar iridescent coloration. In addition to structure, pigments localized within the wing scales contribute to non-iridescent coloration (Stavenga et al., 2014). There are many components that work together to mediate and develop wing patterns, such as gene expression patterns, concentration gradients of morphogen signals, and calcium signals. Research has shown that during early pupation, spontaneous Ca^{2+} waves travel within developing pupal wings (Ohno et al., 2015). Ca^{2+} is likely a trigger for the development of eyespots and is upregulated at injury sites which can lead to ectopic eyespot formation (Ozsu et al., 2017). Cytoplasmic Ca^{2+} can change in response to environmental conditions, especially temperature changes (Otaki, 2007). This led me to hypothesize that Ca^{2+} plays a vital role in pattern mediation beyond eyespots and perhaps be a key feature of wing pattern plasticity in mediating wing pattern elements including the amount of light and dark areas within a wing.

The Model: *Vanessa cardui* is a butterfly in the Nymphalid family, it is found across most of Europe, North America, and Asia (Stefanescu *et al*., 2007) and is known for its long distance migrations (Otaki, 1998). Although there are several plants that *Vanessa cardui* can utilize as a host, in the lab *Vanessa cardui* is also commonly raised using a wheat germ based artificial diet. Using *Vanessa cardui* as a model, it is possible to investigate the primary mechanisms that give rise to the wing patterns and colors that aid environmental adaptation.

Pharmacological Manipulations: It is possible to investigate the effect that calcium has on butterfly wings by manipulating calcium. One possible approach is by utilizing the drug thapsigargin. Thapsigargin is a well-studied Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) protein inhibitor which inhibits the transport of calcium ions from the cytoplasm into the endoplasmic reticulum resulting in cytosolic increases in calcium (Lytton 1991). Additionally, sodium tungstate has previously been shown to induce phenotypic changes (Otaki, 1998) and can thus be utilized as an effective positive control. The mechanism of sodium tungstate in the butterfly is unknown. Wings have dark, white, and colored areas. Changes observed after treating wings with these compounds are most often related to expansions or decreases in melanized dark areas and white areas.

Methodology:

Rearing and Breeding of *Vanessa cardui:* *Vanessa* eggs were obtained from a local breeder and sets of 40-50 eggs were divided among 16 oz cups with a thin layer of artificial diet on the walls. Artificial diet was obtained from Southland Products inc. Larvae were allowed to grow until 2nd instar in 16oz cups and then divided into groups of 5 per 16 oz cup. After the second instar, larvae were fed 2cm² cubes of artificial diets until pupation. Healthy pupae were randomly assigned to DMSO control, Thapsigargin, Tungstate or Coinjection of Thapsigargin and Tungstate treatment groups. After experimental manipulation, pupae were hanged in netting cages, kept out of light, misted daily and allowed to emerge normally.

Specimen Preparation: Upon emergence, specimens were allowed to fully expand their wings and expel meconium before being labeled and transferred to a refrigerator and then to a freezer when ready for processing. Pupae that did not emerge were disposed of after 14 days and recorded. Specimens were processed by pinning specimens on a 4 by 12-inch cork Lepidoptera pinning board. Fore wings were positioned until anal veins were perpendicular to the body and hindwings were allowed to rest beneath. Once positioned, wings were covered with strips of 90 gsm vellum and allowed to dry for at least 14 days.

Manipulating pattern plasticity with pharmacological agents: Thapsigargin and tungstate were delivered into the pupal wing through injections between the anterior pupal wing subcosta vein and the thorax soon after pupation (less than 3H AP) using a 0.3 mm glass capillary hand pulled to a tapered point. The side on which each specimen received an injection was recorded as the ipsilateral and the side opposite to the injection was recorded as the contralateral side. Thapsigargin (1mM) was dissolved in DMSO as it has low solubility in distilled water (Wictome 1992). Sodium tungstate (1M) was dissolved in distilled water. The thapsigargin and tungstate solution were respectively of concentrations 1mM and 1M. Injections of 1µL were delivered in the dorsal basal wing segment using Hamilton 10µL microsyringes mounted with a glass capillary needle. Control specimens were injected with 1µL DMSO. Distilled water injected controls were deemed unnecessary as it has previously been shown that distilled water injections do not result in wing modifications (Dhungel 2009, Colino 2018).

Specimen digitization: All ventral sides of specimens were photographed using a Canon PowerShot SX60 HS in one sitting on a gray (rgb 182, 182, 180) background using a LED light box for consistent lighting conditions. After digitization the hindwings were cropped and assigned an identification number based on treatment.

Quantification procedure: Utilizing ImageJ software, a procedure was developed to quantify black and white wing areas by utilizing color selection thresholds to measure the area and then standardizing the area by converting it to a percent of wing area. First, a color threshold was set by selecting Analyze, then Set Measurement, and selecting checkboxes for: area, mean gray value, and limit to threshold. To measure the area, the hindwing image was opened and then image, adjust and color threshold selected. Using the threshold selection window, black, white and whole wing areas were quantified as shown in Figure 4. HSB color space thresholds were assigned to select for two wing brightness elements: 1. The white to light beige coloration found on *V. cardui* wing webbing pattern surrounding the basal symmetry system, discalis spots and other wing areas. 2. The black discalis, basal symmetry system and ocelli on the wing. Using the ImageJ software default thresholding method in a HSB color space, the respective threshold values ranging from 0 to 255 were used to quantify black, white, and total wing area. Black: hue (0-255), saturation (0-255), and brightness (0-80). White: hue (0-255), saturation (0-55), and brightness (160-246). Wing area: hue (0-255), saturation (0-255), and brightness (0-254).

Statistical Analyses:

Specimen data was processed using GraphPad Prism 9.3.1. Data was first analyzed to determine normality of distribution. Paired data with ipsilateral and contralateral wings was analyzed using parametric paired t-tests. Unpaired data was analyzed by performing a one-way ANOVA with Dunnett's T3 test for multiple comparisons, comparing each treatment group to the control. All p values $p<0.05$ were considered statistically significant. Similarity between the ipsilateral and contralateral wing was confirmed by manually calculating the intraclass correlation coefficient (ICC) from ANOVA outputs.

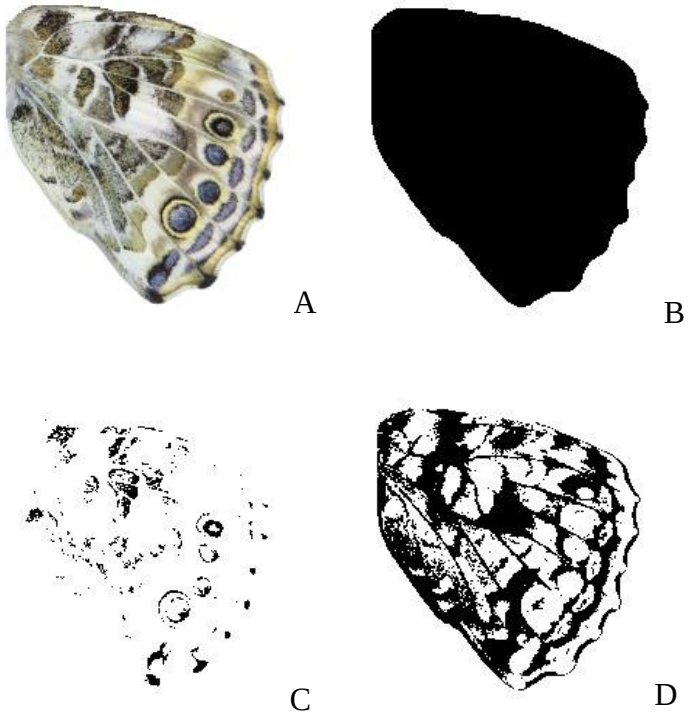


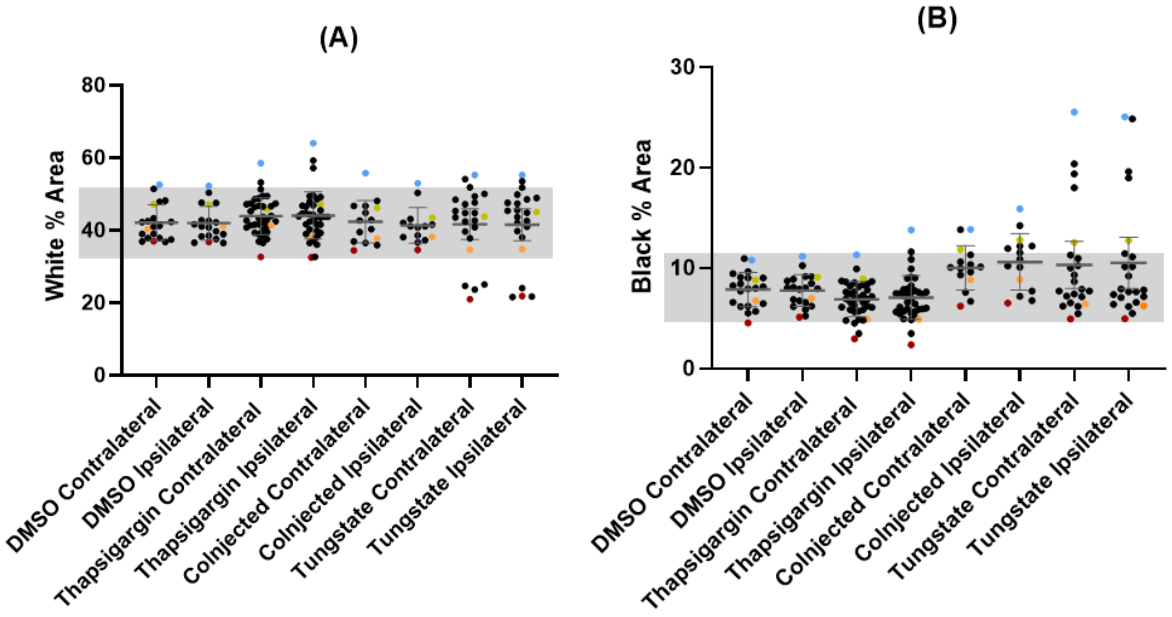
Figure 4. Hindwing ventral quantification binary examples. Original digital image of hindwing, under standardized lighting conditions (A). Binary masks of the whole wing area (B), black wing area (C), and white area (D), according to standardized thresholds.

Results:

Specimen mortality varied across different treatment groups. Of 20 DMSO injected specimens 20 survived to emergence. Of 60 thapsigargin specimens 39 survived to emergence. Of 19 specimens coinjected with both drugs 15 survived to emergence. Of 27 specimens injected with tungstate 23 survived.

Across different treatments there were a variety of brightness changes across wings. Wing changes involved lightening of wings, overall melanization, fading, distortion in eyespots and other pattern elements. Due to the variability of pattern element modifications, quantification of black and white area was an effective method for comparison between specimens and treatments since all specimens possessed black and white area. Distortions in wing pattern elements also result in changes in white and black areas making it a reasonable general representation of overall wing pattern changes. Additionally, the adaptive significance of wing darkness for diurnal Lepidoptera and the previous work with tungstate showing changes in melanization both make melanization a focal element in analysis of phenotypic changes. Wings were also quantified as percentages of total area, to allow for comparisons of white/black areas across specimen wings of different sizes or shapes.

Natural variability and control variability: Although the patterns of *Vanessa cardui* hindwings are very stereotyped, there is some natural variability in the distribution of pattern elements. This natural variability is represented by the percent white area and percent black area data points from control DMSO injected specimen wing data (figure 6). Most treated wings fell within the natural variability (95% confidence interval of DMSO treated wings) but some wing modifications in white or black areas fell outside of this confidence interval of DMSO variability.



(C) Specimen Hindwing Exemplars				
Exemplar Dot Color	DMSO			
	White Area		Black Area	
	Contralateral Wings	Ipsilateral Wings	Contralateral Wings	Ipsilateral Wings
Blue				

Yellow				
Orange				
Red				

	Thapsigargin			
	Contralateral Wings	Ipsilateral Wings	Contralateral Wings	Ipsilateral Wings
Blue				
Yellow				

Orange				
Red				

	Thapsigargin and Tungstate (Coinjected)			
	Contralateral Wings	Ipsilateral Wings	Contralateral Wings	Ipsilateral Wings
Blue				
Yellow				
Orange				

Red				

Tungstate				
	Contralateral Wings	Ipsilateral Wings	Contralateral Wings	Ipsilateral Wings
Blue				
Yellow				
Orange				

Red				

Figure 5. Black and white wing area comparison across all groups. White (A) and black (B) wing areas for all treatment groups. Colored data points were chosen as exemplars (C), to represent wings with the highest value (blue), upper intermediate (yellow), lower intermediate (orange), and lowest value (red) in each treatment group. Gray rectangles represent the 95% confidence interval (CI) of the control datasets: CI for control white area = 32.266 - 51.914 %, and CI for control black area = 4.563 - 11.451 %. Data points outside of these CIs are considered as wings falling oustside the range of DMSO variability. Error bars represent standard deviation. Asterisks represent statistical differences between drug injected groups and the DMSO control (* $p<0.05$, ** $p<0.01$). Statistical comparisons were performed using a one-way ANOVA with Dunnett's T3 test for multiple comparisons, comparing each treatment group to the control. Significant difference in Control vs. Ipsilateral CoInjected black area (P-value: 0.0082) and Control vs. Contralateral CoInjected black area (P-value: 0.0103) comparisons. All other comparisons of black and white area treatment group means were not significant.

Control specimens had higher specimen-to-specimen variation than within specimen variation. The ipsilateral DMSO injected specimen with the greatest white area had 15.8% more white than the specimen with the least white area. Meanwhile, the blackest specimen had 6.4% more black than the least black specimen. The wing differences between the hindwing pairs of

each specimen was approximately 0.1%. The minimal variation within specimens despite larger variation across different specimens is useful for illustrating the importance of comparing within specimen variation (e.g. change in area) and not relying solely on wing area values. The small difference between wings within a wing pair is supported by the intraclass correlation coefficient 0.9893 which suggests high similarity between wings.

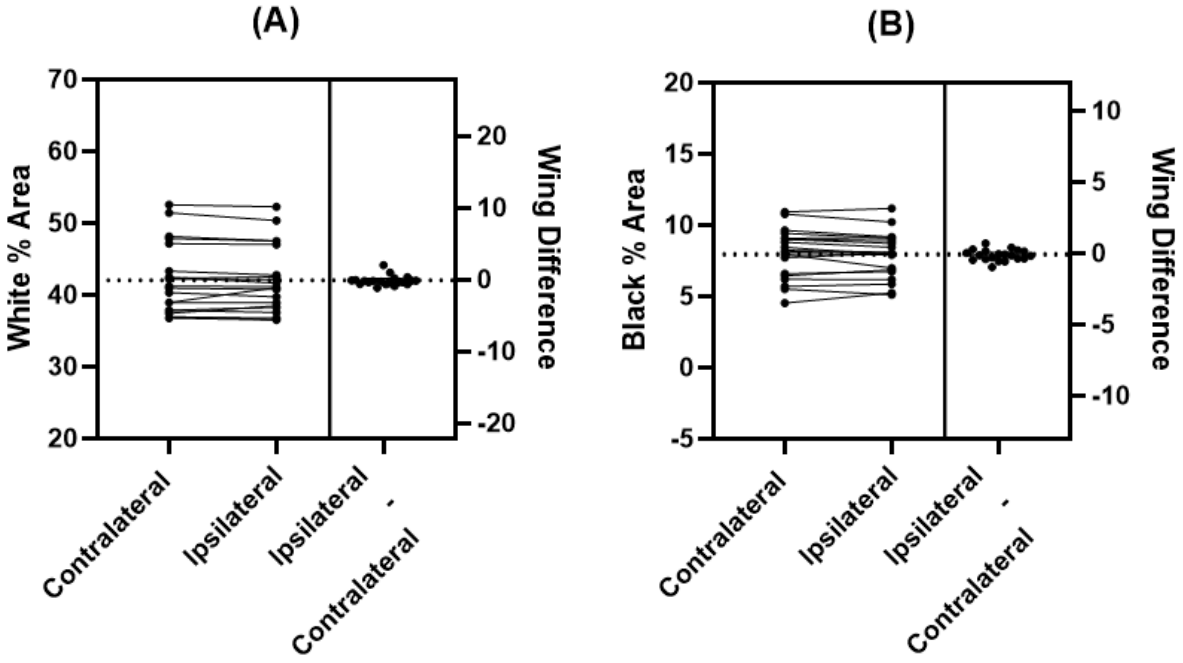


Figure 6. Comparison of white (A) and black (B) areas of DMSO Ipsilateral and DMSO Contralateral wings. Paired data points are from ipsilateral and contralateral wing pairs of the same butterfly. The differences between the paired values are plotted on the right, with mean and standard deviation. Two tailed paired t-tests indicated the wing difference to be not significant. The intraclass correlation coefficient was 0.9893.

Thapsigargin injection results in phenotypic variation. Overall analysis of specimens in figure 6 reveals that pupae injected with thapsigargin showed increased whitening or darkening (examples shown in figure 7). Most wings fell within the baseline variability with some falling

outside the confidence interval of DMSO variation in both ipsilateral and contralateral wings. The following wings fell outside the DMSO variability: The thapsigargin contralateral group had 2 white areas above and 3 black areas below the 95% confidence interval. The ipsilateral thapsigargin group had 3 white areas and 2 black areas above, 2 black areas below the 95% confidence interval. Among these wings falling outside DMSO variability, the more extreme thapsigargin modifications resulted in wing lightening. Although there were cases of darkening, based on qualitative assessment of such specimens (Fig.5 C thapsigargin ipsilateral blue dot exemplar) it can be noted that such darkening phenotypes resulted from distortion-like modifications of the discalis 1 elements. Thapsigargin ipsilateral specimens showed general lightning in the hindwing regions (Fig.5 C, ipsilateral wing, white area, blue dot exemplar) and pattern distortions (Fig.5 C, ipsilateral wing, black area, yellow dot exemplar) of the eye spot.

Thapsigargin wing pairs differed between their ipsilateral and contralateral wings. Paired data in figure 7 shows white and black areas differ between ipsilateral and contralateral wings within the same specimen. This indicates that there was a different modification of black and white in the ipsilateral wing when compared to the contralateral wing. A total of 22 specimens out of 39 showed an increase of white area with a decrease in black area or vice versa.

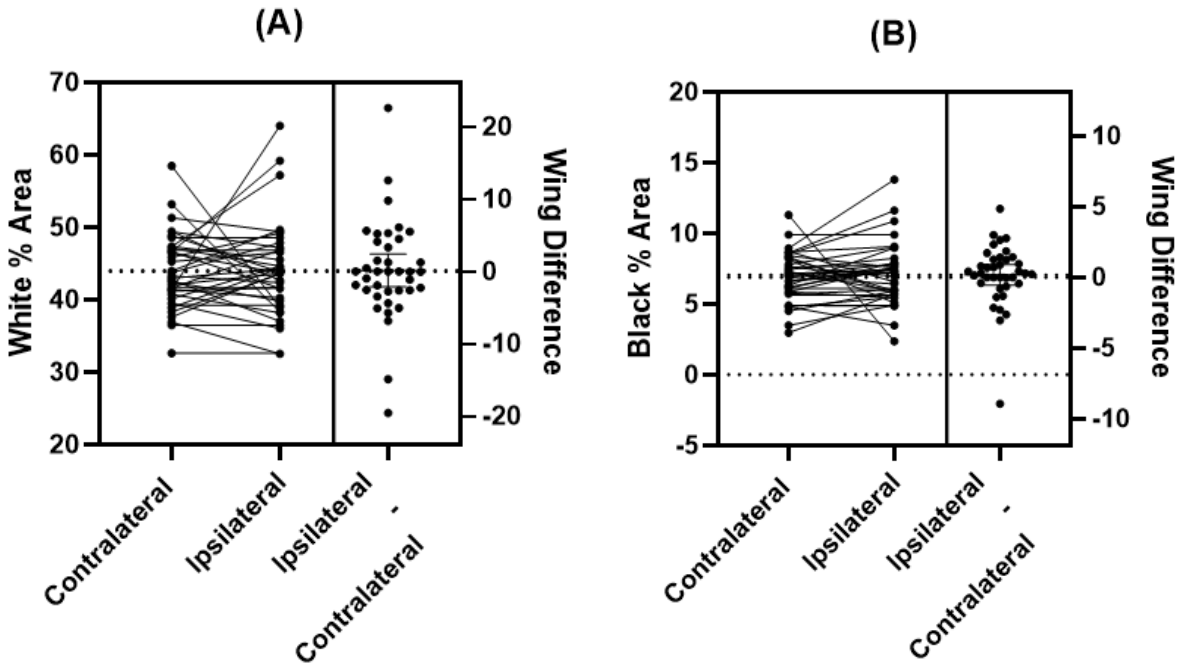


Figure 7. Black and white area change in thapsigargin injected specimens.

Paired data points are from ipsilateral and contralateral wing pairs of the same butterfly. The wing differences between the paired values are plotted on the right, with mean and standard deviation. Two tailed paired t-tests indicated the wing difference to be not significant. The intraclass correlation coefficient was 0.3112.

Tungstate can result in white area increase or extreme dark area increase. Injection of tungstate gave rise to phenotypes with mostly dark area increase with a few white area increases within the baseline variability (Fig.5 A). Out of 25 tungstate injected specimens, 4 exhibited increased black area (Fig.5 C tungstate, white area, red dot exemplar). Overall, tungstate can give rise to an increase in white area and in extreme cases can result in drastic increases in black area. Tungstate paired data (Fig.8) showcases low variation between wing pairs but high variation across specimens. The intraclass correlation coefficient of 0.9962 suggests that ipsilateral and contralateral were both modified very similarly to one another.

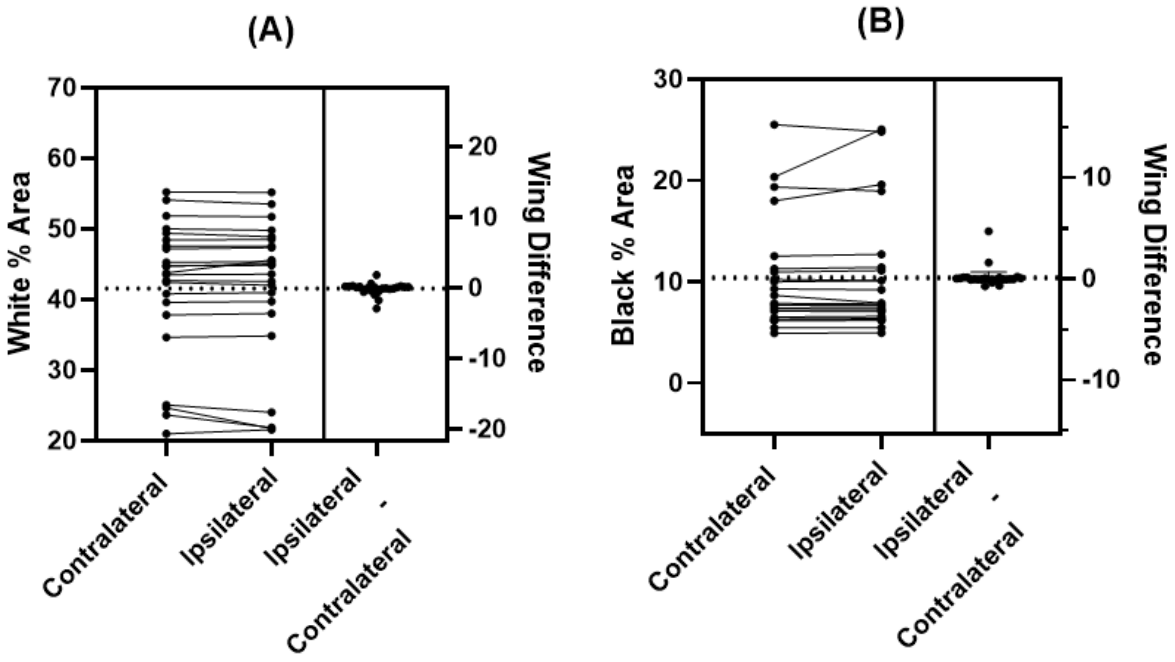


Figure 8. Comparison between contralateral and ipsilateral tungstate injected wing black and white area. Paired data points are from ipsilateral and contralateral wing pairs of the same butterfly. The differences between the paired values are plotted on the right, with mean and standard deviation. Two tailed paired t-tests indicated the wing difference to be not significant. The intraclass correlation coefficient was 0.9962.

Specimens coinjected with thapsigargin and tungstate have an intermediate phenotype between showing characteristics of both treatments. Specimens coinjected with thapsigargin and tungstate exhibited an intermediate phenotype between that of thapsigargin and tungstate. Most wings fell within the baseline variability with some falling outside DMSO 95% CI variability in both ipsilateral and contralateral wings. The contralateral wings had 1 white area above and 3 black areas above the 95% confidence interval. The ipsilateral wings had 1 white area and 7 black area above the 95% confidence interval. The white area was intermediary with that of thapsigargin and tungstate white areas. In general, coinjected specimens had a greater black area than

thapsigargin specimens and less black area than tungstate injected wings outside the black DMSO variability. Contralateral wings and ipsilateral wings were both significantly darker than the baseline variability with respective P-values 0.0082 and 0.0103. Similar to thapsigargin, 8 out of 15 coinjected specimens exhibited increases in black accompanied by decreases white areas of hind wings and vice versa.

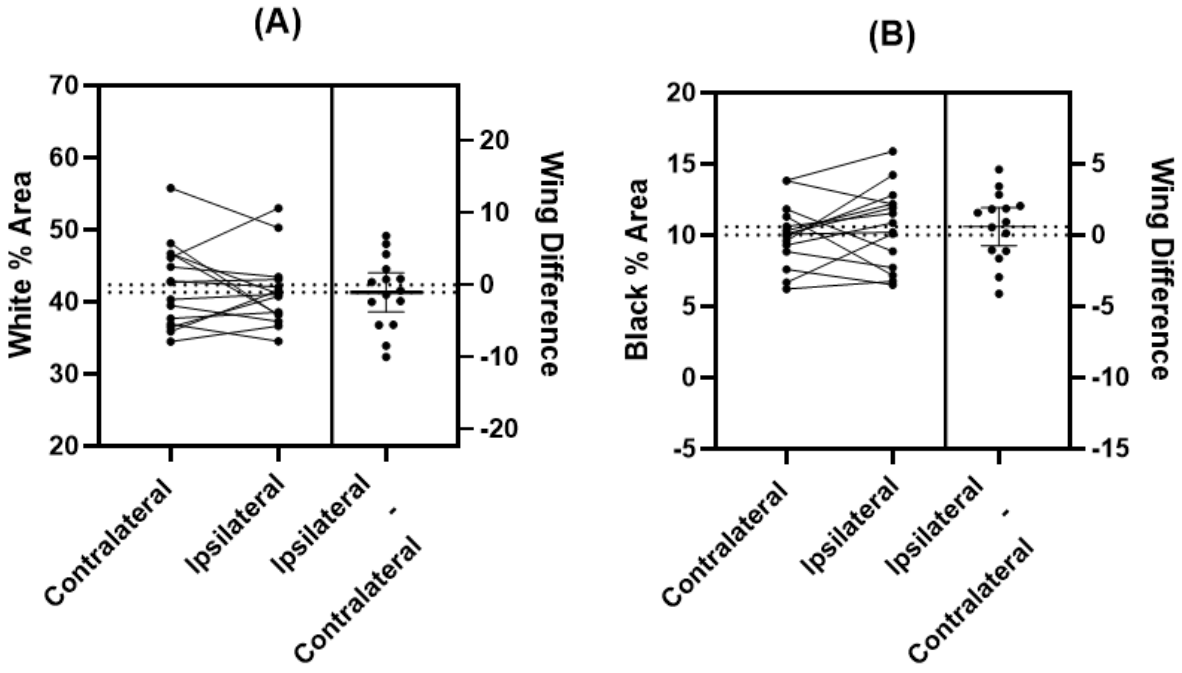


Figure 9. Comparison between contralateral and ipsilateral coinjected wing black and white area. Paired data points are from ipsilateral and contralateral wing pairs of the same butterfly. The differences between the paired values are plotted on the right, with mean and standard deviation. Two tailed paired t-tests indicated the wing difference to be not significant. The intraclass correlation coefficient was 0.5863.

Discussion:

Treatment specific modifications: The two wing modifying compounds used, thapsigargin and tungstate, have differing effects on butterfly wings. Most notably, thapsigargin affects the ipsilateral side of the butterfly differently than the contralateral side but the means are the same. Coinjected specimens injected with both compounds also showed modification on both sides. Tungstate showed symmetrical modifications in both wings. Additionally, mortality with thapsigargin injection was greater than that of tungstate suggesting that thapsigargin has a greater toxicity or impacts a pathway critical for survival.

Control DMSO specimens had high specimen to specimen variation but low within specimen variation. The difference in DMSO injected white and black area between the ipsilateral and contralateral wings (Fig.6) is small, suggesting the wings to be symmetrical. There is a larger variation across wing pairs which showcases specimen variation. This means that the left wing and the right wing of a butterfly are nearly replicates or very close to one another since butterflies have a plane of symmetry. Additionally, the similarity between ipsilateral and contralateral wings suggests that the injection itself and any potential damage does not cause wing phenotype changes. DMSO ipsilateral specimens were used as a baseline for variability in analysis of other treatments.

Specimen means across treatments were not significantly different. Individuals outside the DMSO range of variation in different treatments potentially highlight differences in how each compound affects the wing. Control specimen comparison between the black and white areas of either wing confirms consistency between both wings while also illustrating variation between the white and black areas of individual butterfly specimens. These control wings are considered as a

baseline variability. Treatment with thapsigargin induced variations but the difference between the means of ipsilateral and contralateral wings did not significantly vary. This is likely a result of most wings not being significantly changed but by also looking at wings outside the range of DMSO variability it is possible to understand how the wings are affected by each treatment.

Thapsigargin induces asymmetric changes in injected specimens while tungstate induces symmetric changes in both wings. Thapsigargin induced modifications that varied between the ipsilateral and contralateral wings. Tungstate induced modifications had similar ipsilateral and contralateral wings. Meanwhile coinjected specimens had greater similarity between the wing pairs in comparison to thapsigargin but less similarity than tungstate specimens. DMSO specimens had high similarity comparable to that tungstate. This suggests that DMSO and tungstate both resulted in symmetrical wing pairs and thapsigargin and coinjected specimens had asymmetrical modifications.

Thapsigargin and tungstate effects indicate it may have antagonistic effects within the same or different pathways. Coinjected specimens gave rise to intermediate phenotypes with intermediate black and white areas and modifications correlated with tungstate and thapsigargin injections (figure 9). If acting in the same form within the same pathway, modifications would have been expected to be a cumulation of those of tungstate and thapsigargin. This suggests that both compounds either act in different pathways, antagonistically to one another or opposing one another.

Different pattern development pathways may have potentially been impacted by thapsigargin treatment and may be a subject of future studies. In thapsigargin and coinjected experimental groups, it was observed that thapsigargin modified the ipsilateral wing differently than the contralateral wing within the same butterfly. Thapsigargin ipsilateral and contralateral wings deviated from the controls and exhibited increases or decreases in white area. This is likely due to the different pathways affected. Thapsigargin is a Endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor (Thastrup et al., 1990) which binds to a protein to inhibit it. Hence, thapsigargin is consumed and removed from the hemolymph as it becomes bound, reducing its hemolymph concentration. This potentially explains why it affected one wing differently and why in some cases only part of the wing was modified. Inhibition of SERCA results in cytosolic increases of calcium (Thastrup et al., 1990). Increases of calcium are stress responses by butterflies to a multitude of environmental factors including heat, cold, bacteria and injury (Wood, 2012). Cytosolic increases in calcium may affect localized and delocalized pathways resulting in both localized intracellular effects as well as pupal physiological responses in both wings. This suggests that thapsigargin has a localized direct modification effect on the ipsilateral wing and possibly an indirect effect on both wings.

Direct mechanisms affected by thapsigargin could include WntA or other Wnt pathways. Wnt genes encode ligands involved in Wnt signaling pathways (Thrasivoulou et al., 2013). Such pathways are involved in many development pathways. WntA in particular has been identified as being involved in wing pattern development (Mazo-Vargas et al., 2017). The Wnt pathway involves Ca^{2+} as a secondary messenger. Thapsigargin depletes Ca^{2+} stores of the ER similar to how Wnt ligands induce calcium release in the Wnt/Ca^{2+} pathway. This results in depolarization

of the nuclear envelope and translocation of B-catenin transcription factor (Thrasivoulou et al., 2013). Additionally, Wnt ligands act as morphogens (Mehta et al., 2021) possibly inducing gradients in wing patterning. WntA mutant knock-outs in *Vanessa cardui* have previously been shown to produce phenotypes with loss of patterning between discalis 1 and ocelli elements (Mazo-Vargas et al., 2017) which share similarities to some thapsigargin specimens (Supplemental Figure. Thapsigargin 16, 20, 21 and 26).

Elevated thapsigargin induced calcium levels may have distorted wing eyespots. The effects of thapsigargin on eyespots are a potential area of future study. Thapsigargin injections resulted in eyespot distortions (exemplars in Supplemental Figure. Thapsigargin 1, 10, 19, 23 and 35) and complete loss of eyespots (exemplars in Supplemental Figure. Thapsigargin 31 and 38). Calcium signaling is an early trigger associated with eyespot development and wound healing (Özsu et al., 2017). It has also been shown that damaging a butterfly's pupal wing can trigger ectopic eyespot formation and Ca2+ increase (Wood, 2012). Hence, it is likely that thapsigargin induced Ca2+ increases could influence eyespot development and distort or terminate eyespot development just as injury and damage has been shown to (Otaki, 2018).

Scale microstructures may have been impacted by ER stress reducing scale pigment intensity. Several thapsigargin treated specimens also exhibited bleaching-like events where the intensities of the pigments decreased. This is different from increase in white area as increases in white area involve the absence of pigment where bleaching involves the decrease in the intensity of the pigment. It is possible that calcium impacted the development of the pigments but decreases in microstructural iridescent blue color from the eyespots of regions affected by “bleaching”

(exemplars in Sup. Thapsigargin 13, 17, 20, 21, 27 and 30) suggest the possibility that microstructural modifications occurred. Scales are made of chitin and some enzymes involved in the formation of chitin in insects are secreted from the endoplasmic reticulum (Merzendorfer, 2003). Given that thapsigargin directly targets the ER by draining calcium stores it is possible that the production or mobilization of chitin related enzymes is affected by thapsigargin injections leading to scale structure deformities that reduce the visual intensities of the colors. Microscopy would be necessary to confirm if bleached scale microstructures differ from regular scales microstructures.

Thapsigargin could also impact temperature sensitive proteins. One example of a gene whose product could potentially be impacted by thapsigargin is *Painless.* The gene *Painless* encodes a transient receptor potential ion channel family protein and is involved in responses to noxious heat stimuli in the presence of calcium (Tracey, 2003). *Painless* has also been shown to be expressed in butterfly eyespots (Özsu, 2017) which suggest a possible genetic connection between calcium and heat responses.

Indirect effects are possibly a result of general stress responses and factor secretion. In particular stress responses (Adamo, 2017) and temperature shock responses (Zhao et al., 2012)) can sometimes result in phenotypic changes (Luo, 2015). Usually pupal environmental stress responses elicit symmetrical modifications (e.g same changes in both wings) but in thapsigargin injected specimens localized effects trigger location specific modifications while general effects may give rise to overall changes in both wings.

Thapsigargin may modify in a time sensitive manner related to pathway activity. These different pathways are present during different stages of development (Reed, 2005) and depending on when thapsigargin is injected there could be a different assortment of pathways affected. For example, injection before melanization could result in black area being affected and absence or reduced black wing area while injection during melanization could result in distortions of the black areas or increases of black area.

Tungstate also induced modifications of specific features. Some tungstate injected specimens (exemplars in Supplemental Figure. Tungstate 1, 2, 8, 18, 20, 22) exhibited changes in the ratio of blue iridescent coloration, black, white and yellow within eyespots. Some specimens injected with tungstate that exhibited extreme melanization also had eyespots go completely white (exemplars in Supplemental Figure. Tungstate 1, 18, 20) and begin fading and decreasing in eyespot diameter (exemplars in Supplemental Figure. Tungstate 20, 18). In one hypermelanized specimen the eyespots remained and appeared to have blue filling typically black area within the eyespot (Supplemental Figure. Tungstate 8). In other cases eyespots and parfocal elements distance decreased in some cases appeared to begin to fuse (Supplemental Figure. Tungstate 2, 13, 22, 7 and 15) and in Supplemental Figure. Tungstate 3, some parfocal elements were missing and eyespots had smaller diameters. One specimen (Supplemental Figure. Tungstate 9) had almost complete absence of eyespots and distortions in the wing pattern. Tungstate injections in butterflies have been investigated extensively and this experiment's results for tungstate injections did not differ drastically from previously reported findings (Dhungel et al., 2009. Otaki et al., 2005. Mahdi et al., 2010). Tungstate has been hypothesized to act as a protein tyrosine phosphatase (PTPase) inhibitor (Otaki, 2004). Based on this, tungstate may function by preventing signaling to scale

forming cells. Variability of tungstate dose received by individual scale forming cells and the timing of injection would give rise to variable modifications (Terzin, 2014). Despite this, the mechanism of tungstate has yet to be confirmed.

Additional factors that possibly contributed to variation in modification across specimens include dose dependence and timing of injection. Although injections of drugs were delivered as consistently as possible, we cannot account for additional factors like variation in specimen size and drug slippage. Specimen size could have resulted in smaller specimens receiving a higher dose to body mass ratio resulting in possibly greater modification effects in smaller specimens. Drug slippage occurs as a result of hemolymph bleeding, as specimens are being injected, some hemolymph may leave through the injection hole in pupae and as a result expel varying amounts of drug. Additionally, based on the 3-hour specimen injection window, it is possible different wing development pathways were affected based on the specific timing of injection.

Based on the wide range of modifications that resulted from drug injections that may have arisen from time of injection, future studies may be able to investigate time specific wing modifications within this study's 3-hour period of injection. One possible method would be to use time lapse photography to create a standard curve of pupal cuticle hardening over time that can be used to determine exact timing of drug injection. Additionally, using genetic approaches to target genes that encode proteins involved with calcium could offer a more direct approach at investigating calcium and its associated proteins.

Conclusion

Since thapsigargin is well known to cause a sudden increase of cytosolic Ca^{2+} ions by inhibiting the SERCA Ca^{2+} pump in many systems, these phenotypic changes support the hypothesis that Ca^{2+} plays an important role in wing brightness mediation, particularly in the formation of black areas (melanin) and wing brightness. Although the mechanism of action for tungstate is unknown, the difference of tungstate to thapsigargin-induced effects, suggest that tungstate acts in a different pathway than that of thapsigargin or has opposite effects within the same pathway. Modifications induced through pharmacological manipulations of Ca^{2+} levels suggest that one or more Ca^{2+} signaling related pathways may underlie wing phenotypic plasticity.

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**Thesis and supplemental figures can be accessed through scholarworks.iu.edu under the title Painting Wings - Evidence for a Role of Ca^{2+} in Butterfly Wing Phenotypic Plasticity **

Supplemental Figures:

	Total Number Injected	Specimen Survival Number	**B-/W+**	**B+/W-**	**E-**	**E+**	**B+/0**	**B-/0**	**0/W+**	**0/W-**	**0/0**
DMSO Injected	20	20	0	0	0	0	0	0	0	1	19
Thapsigargin Injected	60	39	9	13	1	2	3	1	3	1	6
Coinjected	19	15	5	3	0	2	4	0	0	1	0
Tungstate Injected	27	23	7	6	0	2	2	0	4	1	1

Supplemental Table 1, modification events in different treatment groups. Wing black or white area modification events for different treatments were seen in 8 different ways: 1) An increase in white area and a decrease in black area [B-/W+]. 2) A decrease in white area and increase in black area [B+/W-]. 3) A decrease in white and black areas of both wings [E-]. 4) An increase in white and black area of both wings [E+]. 5) Less than 1.16% change in white area and increase in black area [B+/0]. 6) Less than 1.16% change in white area and decrease in black area [B-/0]. 7) Increase in white area and less than 0.32% change in black area [0/W+]. 8) A decrease in white area and less than 0.32% change in black area [0/W-]. 9) Less than 0.32% change in black area and less than 1.16% change in white area [0/0]. Thresholds for change significance were set at 1.16% for white area and 0.3% for black area based on the standard error of means of 1.16 and 0.32 for the DMSO injection treatment group. In most but not all wings, black area had an inverse proportional relationship to that of white area where an increase in black area resulted in a decrease in white area and vice versa. A smaller number of wings exhibited increases or decreases in both white and black or only changes in white or black area.

Control DMSO Injected Specimens			
ID#	Increasing Black (Going Down)	ID#	Decreasing White (Going Down)
3		11	
12		3	
11		9	

1		1	
10		20	
2		12	

18		4	
7		16	
16		8	

6		19	
8		10	
14		2	

4		6	
13		17	
19		7	

9		18	
20		15	
5		13	

15		14	
17		5	

	Thapsigargin Specimen Contralateral Wings		
ID #	Increasing Black (Going down)	ID #	Decreasing White (Going Down)
14		14	

2		2	
38		29	
27		1	

29		19	
33		24	
12		32	

32		22	
16		16	
5		13	

28		33	
19		10	
37		21	

24		27	
10		3	
35		17	

25		31	
9		5	
22		9	

7		12	
13		38	
3		7	

21		6	
31		25	
23		20	

6		30	
18		28	
17		23	

11		18	
4		11	
8		15	

34		35	
1		4	
15		26	

20		8	
26		34	
36		37	

30		36	

Thapsigargin Injected Wing Exemplars			
ID #	Increasing Black (Going down)	ID #	Decreasing White (Going Down)
30		30	
27		21	

12		16	
33		31	
2		29	

17		3	
5		32	
29		5	

32		19	
4		20	
14		33	

21		17	
11		22	
38		11	

16		13	
37		4	
9		12	

20		24	
6		8	
22		27	

35		6	
3		26	
13		1	

24		9	
25		23	
28		25	

8		10	
23		28	
7		7	

19		35	
31		14	
10		2	

18		18	
15		38	
36		37	

34		15	
1		36	
26		34	

Thapsigargin and Tungstate Non-Injected Specimens			
	Increasing Black (Going Down)		Decreasing White (Going Down)

9		13	
13		9	
4		10	

10		6	
14		14	
7		12	

2		8	
12		2	
15		7	

11		11	
6		4	
5		5	

8		1	
1		15	
3		3	

Thapsigargin and Tungstate Injected Specimens			
	Increasing Black (Going down)		Decreasing White (Going Down)

4		9	
9		5	
5		4	

10		8	
8		13	
13		10	

12		14	
14		6	
6		2	

15		1	
1		11	
11		3	

2		7	
7		12	
3		15	

Tungstate Injected Ipsilateral Wings			
ID#	Increasing Black (Going down)	ID#	Decreasing White (Going Down)
5		5	
17		15	
11		21	

21		9	
3		12	
12		3	

6		17	
14		23	
19		16	

7		14	
15		2	
23		6	

16		11	
10		7	
9		22	

13		13	
22		19	
4		4	

2		10	
18		8	
20		1	

8		20	
1		18	

Tungstate Injected Contralateral Wings			
ID#	Increasing Black (Going down)	ID#	Decreasing White (Going Down)
5		5	

17		15	
11		21	
21		9	

3		12	
12		3	
6		17	

14		23	
19		16	
7		14	

15		2	
23		6	
16		11	

10		7	
9		22	
13		13	

22		19	
4		4	
2		10	

18		8	
20		1	

8		20	

1		18