

Comparing the pigmentation development in *Drosophila americana* and
novamexicana sister species

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Abstract

Pigmentation studies can be used in order to establish patterns of divergence, and *Drosophila* is a common organism to study the evolution, due to the many species and the ease of following many generations of flies. Here, I study the evolution of two closely related *Drosophila* species, *Drosophila americana* and *Drosophila novamexicana*, by analyzing the genes that are involved in pigmentation, leading to the noticeably different phenotype exhibited by the two species. The goal is to figure out if Tan, a protein that leads to dark brown melanin in the pigmentation pathway, is expressed at different levels in *D. americana* and *D. novamexicana* and to see if the development of the species and the Tan protein in those species differs. I found through in situ hybridization that at the late pupal stage, *tan* mRNA seems to be equally expressed in the two species. I also compared pupal development in *D. americana*, *D. novamexicana*, and *D. melanogaster* and show that *D. americana* and *D. novamexicana* developed at similar rates to each other and that were both slower than that of *D. melanogaster*. Third, I compared expression levels of Tan proteins throughout the development of the three species and look at *D. americana* and *D. novamexicana* in particular. The Westerns suggested that Tan is being expressed much earlier in development of *D. americana* and *D. novamexicana* than expected, and although there was a difference in expression, there was a very different pattern than expected. This could be because the Tan antibody might be binding to something else. From the different tests, I show that there does not seem to be any real indicator of the pigmentation divergence between *D. americana* and *D. novamexicana* through the *tan* mRNA and the development of the species; and that further testing is needed to see if the Tan antibody is binding to the correct protein.

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Introduction

Every living organism, no matter how distantly related, has evolved from a common ancestor. While it is interesting to study how this one organism evolved and formed all of the living things that currently exist, this is a very complicated task. Therefore evolutionary scientists often study species that are more closely related, in order to determine the evolutionary patterns that exist throughout the world. The patterns of evolution of traits between these closer species can then be generalized and used as a basis for analyzing more large-scale evolution. Distantly related organisms can still have some shared traits, and the study of the conservation of these phenotypes can show how these organisms have diverged. By contrast, species that are similar to each other in their genetic code can have very different morphologies. By studying the changes in genes between related species, geneticists can determine how those species have diverged from a common ancestor.

Pigmentation studies can be very useful in the study of evolution since changes in pigmentation genes lead to phenotypes that are easy to analyze and follow throughout the divergence of the organisms. In addition, pigmentation differences are often polygenic and correlate with changes in regulatory genes. So the study of melanization in different species of *Drosophila*, for instance, helps us to understand the genetic and developmental basis for phenotypic diversity (Wittkopp et al 2003). Certain organisms, such as flies in the *Drosophila* genus, are more useful than others in evolutionary studies. Flies in this genus have a short generation time and are small, with large numbers of progeny. Therefore, it's very easy to work with the organism and record the changes in pigmentation throughout generations. In addition, there are many different *Drosophila*

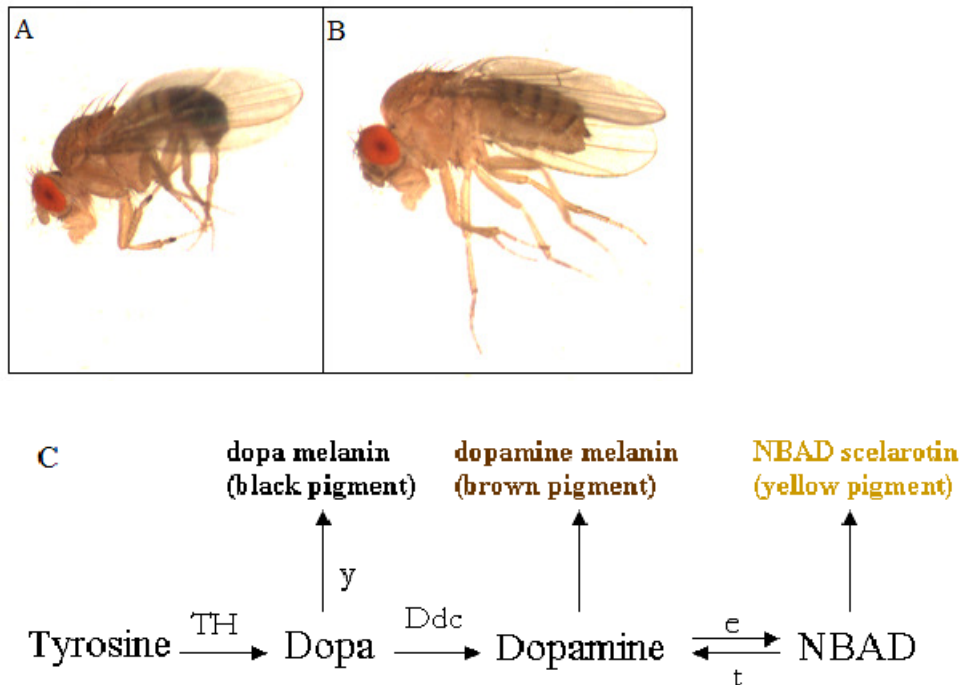
species from which scientists can study evolution. The genome of *Drosophila melanogaster* has been sequenced in its entirety, and in fact this past year, the genomes of twelve other species of *Drosophila* have also been successfully sequenced (Clark et al 2007). This provides scientists with an easy way of finding already existing mutations for genes, in order to see the genetic and adaptive response to such mutations.

Due to the convenience of using pigmentation genes in evolutionary studies, along with the availability of sequenced mutations in *Drosophila*, many scientists have studied pigmentation in related *Drosophila* species to show their evolution. For example, Brisson et al (2006) used pigmentation markers in order to study evolution of species in the *Drosophila cardini* group that are geographically isolated. They showed through pigmentation studies that the path of evolution was from one common ancestor, but that it did not follow a simple evolutionary model (Brisson et al 2006). In a similar study, Hollocher et al (2000) scored abdominal pigmentation in species belonging to the *Drosophila dunni* subgroup. The proposed evolutionary model of a series of repressors and activators that map to the pigmentation differences also translates into a phylogeny model for the subgroup (Hollocher et al 2000; Hollocher et al 2000). In these examples, pigmentation genes were used in order to make generalizations about overall evolution of related species, thus showing that studying the difference in pigmentation gene expression in *Drosophila* can be useful in providing a basis for further evolutionary study.

The Pigmentation Pathway in *Drosophila*

In order to study the evolution of *Drosophila* species through the difference in pigmentation genes, it is important to first understand the genes involved in the pathway to melanization. As shown in Figure 1, the general melanin pattern for *Drosophila melanogaster* is alternating light and dark pigments, mostly black or brown with a lighter tan or yellow background (Wittkopp et al 2002). The pigmentation biosynthesis pathway as shown has been proposed by Wittkopp et al (2002).

Figure 1. Difference in Abdominal Pigmentation of *Drosophila melanogaster* Males and Females and the *Drosophila* Pigmentation Pathway



(A) Male adult *D. melanogaster* flies show solid dark pigmentation in the last abdominal segments while in (B), female adult *D. melanogaster* flies retain the striped pattern throughout the abdomen. (C) shows the proposed biochemical pathway for pigmentation in *Drosophila*

In order to form different melanin patterns, the pigmentation pathway has different possible pigmentation outcomes. In the pathway, shown above in Figure 1C, tyrosine is converted by Tyrosine Hydroxylase to dopa, and then dopa becomes

dopamine with the help of Dopa Decarboxylase, and both dopa and dopamine lead to different melanin, using phenyl oxidases (Wright 1987). Next, dopamine can be converted to N- β -alanyldopamine, which then leads to a third type of pigment. There are three main essential genes involved in the melanization pathway that have been identified: *yellow*, *tan*, and *ebony*. These genes lead to black, brown, and yellow melanin, respectively, coming from dopa, dopamine, and NBAD; and they have also been shown to have other functions in the fly as well.

Once researchers determined the important proteins involved in the pigmentation pathway, the next step was to figure out how these proteins work together to form the melanin. While studying the reciprocal functions of Yellow and Ebony, Wittkopp et al (2002) found that Ebony suppresses the melanin formation due to Yellow, leading to the yellow pigmentation due to Ebony. Ectopic *yellow* over expresses the protein, inducing the formation of black melanin when Ebony is not present, and ectopic *ebony* will inhibit melanin formation, reversed by adding *yellow* (Wittkopp et al 2002). So Wittkopp et al show that the two proteins have a reciprocal function in the pigmentation pathway. In addition, studies of the *tan* gene show that *tan* and *ebony* work in a reciprocal manner in order to produce the appropriate pigment (L. Arnold, unpublished data). True et al (2005) have proposed two main functions for *tan* in *Drosophila*: one is the function of the gene in the melanization pathway, where the Tan protein hydrolyzes NBAD to form dopamine, and the other is the hydrolysis of carcinine to histamine in the formation of the photoreceptors. In the pigmentation pathway Ebony encodes NBAD synthetase, which converts dopamine back to NBAD while Tan hydrolyzes NBAD to dopamine (Wright 1987). If Ebony is expressed, then NBAD forms the lighter, yellow-colored melanin and

if Tan is expressed then NBAD will mostly turn back into dopamine, which forms the brown pigment. The so-called Ebony-Tan shunt is important in pigmentation formation, because the production of dopamine depends on Tan, which depends on Ebony for its substrate, thus making *ebony* epistatic to *tan* (True et al 2005). So in *Drosophila* there is an epistatic, reciprocal relationship between Ebony and Tan, just as there is a reciprocal relationship between Ebony and Yellow in the pigmentation pathway. Therefore the genes in the pathway seem to be connected, and studying these genes should provide reasons for the differences in pigmentation between *Drosophila* species. By studying the genes involved in the pigmentation pathway, researchers can study the evolution of melanism in *Drosophila*.

Once these pigmentation genes were analyzed and named, they could be used for studying evolution of similar species. Llopart et al (2002) studied the difference between *Drosophila yakuba* and *Drosophila santomea*, two related species with very different pigmentation. *D. santomea* is characterized as having a loss of pigmentation in the lower abdominal regions, unlike *D. yakuba*, which retains that pigment. The study suggests that 90% of these differences in pigmentation occurred due to the X chromosome, and the researchers were able to sequence nine different non-coding regions of each species in order to estimate a divergence time of around 400,000 years ago (Llopart et al 2002). Pigmentation studies were then able to further increase the knowledge of the evolution of the two species. In a study by Jeong et al in 2008, the expression of *tan* and *yellow* was shown to correspond to the divergence of pigmentation between *D. yakuba* and *D. santomea*. When studying the genes, it was shown that expression of *tan* is lost in *Drosophila santomea* through CRE specific mutations but the sequence of Tan protein

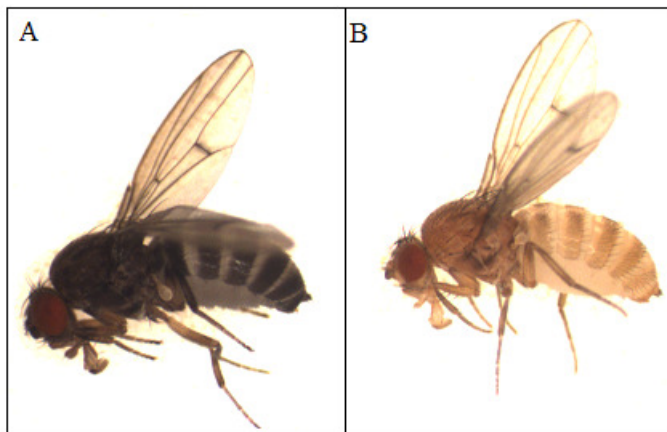
doesn't change (Jeong et al 2008). Jeong et al (2008) constructed a *D. melanogaster tan* transgene by covering the *tan* locus by constructs and then making a shorter rescue construct in the noncoding region between two annotated genes that were spanned by the original, larger constructs. By inserting this *D. melanogaster* transgene into *D. santomea* flies, the pigmentation in *D. santomea* was partially restored (Jeong et al 2008), thus implying that selective loss of *tan* and *yellow* led to the loss of pigmentation in *D. santomea*. These two papers then show how pigmentation markers can be used to give insight to overall evolution of related species.

Drosophila americana* and *Drosophila novamexicana

Similar to *Drosophila yakuba* and *Drosophila santomea*, there are other related species in the genus that can also be studied using the pigmentation genes. Two such sister species are *Drosophila americana* and *Drosophila novamexicana*, two members of the *Drosophila virilis* species group, with the closest relative to the two being *D. virilis* (Wittkopp et al 2003). The divergence time for *D. virilis* and *D. melanogaster* is around 65 million years (Wittkopp et al 2002), so *D. americana* and *D. novamexicana* have a long divergence time from *D. melanogaster*. Spicer used 2-dimensional electrophoresis in 1991 to analyze the twelve species in the *D. virilis* species group and found that *D. americana* and *D. novamexicana* diverged from a common ancestor only around five million years ago (Spicer 1991). This divergence time was narrowed down even further by Vieira et al in 1997 to be only around four million years. Then a recent study from 2008 showed that *D. americana* and *D. novamexicana* have diverged around 300,000-500,000 years ago (Morales-Hojas et al 2008). The two species are closely related

enough to be capable of interbreeding to form hybrids (Nurminsky et al 1996; Wittkopp et al 2003). Despite the evolutionary closeness and genetic similarities, however, the two species are quite different from each other morphologically. Wittkopp et al (2003) found that the dorsal thorax of *D. americana* is more pigmented and has a much darker melanin than that of *D. novamexicana*, and the same is true in the abdomen. In contrast, the overall abdominal cuticle color of *D. novamexicana* is much lighter than that of *D. americana*, containing more of a yellow or light tan pigmentation while the abdominal cuticle of *D. americana* is a much darker black or tan color (shown in Figure 2)

Figure 2. Pigmentation Differences Between Adult *D. americana* and *D. novamexicana*



(A) A fully pigmented *D. americana* adult female shows dark pigmentation throughout the thorax and abdomen. (B) A fully pigmented *D. novamexicana* adult female shows a light tan/yellow pigmentation throughout the thorax and abdomen.

Wittkopp et al (2003) also performed hybrid crosses on the flies to see the effect on future generations. When these strains were crossed, the hybrid generation of flies had a dark thorax and abdomen due to the contribution of pigmentation genes from *D. americana* but a yellowish overall color, due to influences in pigmentation genes from *D. novamexicana* (Wittkopp et al 2003). This mixture of expression levels from the two parent species in the hybrids suggest that the difference in pigmentation between *D. americana* and *D. novamexicana* is a genetic trait, since it was passed on to the next

generation. Therefore *D. novamexicana* and *D. americana* appear to be ideal for studying the pigmentation genes, in order to help explain the extreme morphological divergence in the short period of evolutionary time.

Expression of Pigmentation Genes in *D. americana* and *D. novamexicana*

Analysis of Yellow and Ebony by Wittkopp et al (2003) showed no noticeable difference in the expression of Yellow for *D. americana* and *D. novamexicana*, despite what might be expected with their considerable morphological differences. In this paper from 2003, Wittkopp et al found significant linkage with *ebony* and the different cuticle pigmentation in the abdomens, though no such association was found for *yellow* (Wittkopp et al 2003). The hybrids from the paper seemed to show no maternal effects, though the pigment was shown to resemble the female parent slightly more, implying that at least one pigmentation gene is located on the X chromosome (Wittkopp et al 2003). From the lack of significant association between Yellow expression and pigmentation, it seems that the pigmentation gene on the X chromosome must be *tan*, and this was in fact proven to be true by genetic associations (True et al 2005). Ebony is responsible for lighter melanin but does not explain the reason for the darker pigmentation as seen in *D. americana*, so it is assumed then that Tan must also be expressed differently in *D. americana* and *D. novamexicana*. The specific biochemical function of Yellow in the pigmentation pathways is still unclear, but the Yellow protein, along with Tan, seems to be responsible for the darker pigmentation; and it has been shown that Yellow forms black pigmentation in the larval and adult cuticle, and the *yellow* or *y* gene shows a strong correlation with the black melanin deposition (Walter et al 1991). Therefore, the lack of

significant difference in Yellow expression suggests that Tan must be playing a differential role in the divergence of pigmentation, and therefore the overall evolution of *D. americana* and *D. novamexicana*.

The hypothesis for this thesis is therefore that there will be a difference in expression levels of *tan* in the abdominal cuticles of the two species. In this experiment, I have investigated the expression of *tan* mRNA and Tan protein levels in *Drosophila americana* and *Drosophila novamexicana*. Through in situ hybridizations I found no discernable difference in mRNA levels for *tan* in the late stage pupae. I also tried to examine Tan protein expression using immunohistochemistry, but was unable to gain conclusive results because of problems with the antibodies being used. I have also studied the developmental progress of the two species, both in the morphology and the expression of Tan throughout those stages, in comparison to *Drosophila melanogaster*, whose developmental time frame has previously been staged. From this data, I found that both *D. americana* and *D. novamexicana* age at a consistently slower rate than *D. melanogaster*; and that there is a higher degree of variability in the staging for those two species when compared to *D. melanogaster*, but that there is no significant difference in the staging of the two. I will show that the Western blots for *D. melanogaster* show that Tan expression peaks right before eclosion, but that the antibody used for Tan in *D. americana* and *D. novamexicana* shows signal starting much earlier in the pupal development. The protein expression levels are already present and strong for both *D. americana* and *D. novamexicana* at the earliest pupal stage being tested in the Western blot series. In addition, the Westerns show a stronger signal in *D. novamexicana* for Tan expression when comparing two wells with approximately equal loading

amounts, in the late pupal stages. Therefore, this suggests that either Tan has an unexpected function in *D. americana* and *D. novamexicana*, or else, the antibody is actually binding to some protein other than Tan.

Comparison of *tan* Expression at Late Pupal Stages

As mentioned earlier, *Drosophila novamexicana* and *Drosophila americana* are two closely related species with strong differences in their morphology, making them excellent subjects for genetic research. Previous studies involving the pigmentation pathway genes of *D. americana* and *D. novamexicana* have shown that there is a correlation between Ebony expression and the differences in pigmentation between the two species. However there is no visible difference in the expression of Yellow between those same species, and so this does not help account for the darker pigmentation as seen in *Drosophila americana*. The same study that found a correlation between *ebony* expression and pigmentation differences also showed that *yellow* is expressed highly in both species of flies, so there doesn't seem to be a strong correlation between *yellow* and the pigmentation divergence (Wittkopp et al 2002). Since Yellow and Tan are both associated with darker melanin, it is likely that the darker pigmentation in *D. americana* is due to a stronger expression level of *tan*, the gene that contributes to dark brown melanin in the pathway.

Preliminary work with the *tan* gene has been conducted by the Wittkopp lab at the University of Michigan, by conducting a fine scale mapping of the *tan* gene and analyzing how *tan* activity leads to various pigmentation categories, through hybrid crosses between *D. americana* and *D. novamexicana*. The crosses of *D. americana* females to *D. novamexicana* males and backcrosses to *D. novamexicana* males showed different pigmentation classes (Wittkopp et al, unpublished data). The male offspring with the *D. novamexicana tan* allele all had lighter pigmentation while the males that inherited the *D. americana tan* allele had darker pigmentation. This suggests that there

are sites on the X-chromosome that are linked to *tan* and contribute to the pigmentation difference seen between the two species. By over-expressing Ebony and Tan, Wittkopp et al (unpublished) found intermediate pigmentation classes as well, suggesting that the proportions of the two proteins determine the brown and yellow pigmentation in *D. americana* and *D. novamexicana*.

By performing the fine scale mapping of *tan*, Wittkopp et al (unpublished data) found two recombination sites of the *tan* gene, one located slightly upstream of the first exon and the other one in the first intron, between the first and second exons. When there were no recombination events and the entire *tan* gene was from the *D. novamexicana* parent, the pigmentation of the cuticle was found to closely resemble that of *Drosophila novamexicana*, while the *D. americana tan* gene without recombination led to normal *D. americana* melanization. From these recombination break points, Wittkopp et al (unpublished data) found a 2.7 kb region in the *tan* gene that includes the first intron, and that seems to contribute to the difference in pigmentation. The region contains insertions and deletions that are different between the two species, and they all affect non-coding sequences (Wittkopp et al, unpublished data). These results, along with the results from the hybrid crosses, suggest that *tan* is being expressed differently in *D. americana* and *D. novamexicana*, since differences of *tan* expression from the two parental flies in a hybrid changes the pigmentation of the resulting hybrid, and because the mapped region in the first intron has so many differences between the two species.

Therefore, we hypothesized that *Drosophila americana* and *Drosophila novamexicana* would exhibit differences in the levels of *tan* mRNA and expression of the Tan protein in the abdomens. Through in situ hybridization, we studied the levels of *tan*

mRNA in the abdominal cuticle of late stage pupae of those two species. We found that, similar to the results of previous studies involving Yellow protein expression in *D. americana* and *D. novamexicana*, there seems to be no difference in levels of *tan* mRNA signal in the late pupal cuticles of those two species, going against the hypothesized results.

Materials and Methods

Drosophila Strains: Wild-type strains of *Drosophila americana* and *Drosophila novamexicana* (strains A00 and N14) were raised on standard cornmeal media at room temperature (i.e., 22-25 degrees Celsius).

In situ tissue preparation: The flies were collected at the late pupal stage, after the wings had turned black and before eclosion. The pupae were heated at 100° C for one minute then dissected to gain only the cuticle. The cuticles were fixed in Phosphate Buffer and Triton-X (PBT) + 4% paraformaldehyde and washed with methanol (MeOH) and ethanol (EtOH), and then stored in EtOH at -20°C until they were used for in situ stainings.

In situ hybridization: Cuticles were washed with EtOH and then incubated in 50:50 xylenes:EtOH. Then the cuticles were washed with EtOH, MeOH, and then PBT, and fixed again in PBT + 4% paraformaldehyde. After washing again with PBT, they incubated with 1:25,000 PBT: Proteinase K. They were washed with PBT, post fixed with PBT + 4% paraformaldehyde, washed again with PBT, then 50:50 PBT:Hybridizing (Hyb) solution, and Hyb solution, and then Hyb solution and incubated with the probe overnight at 65°C. The next day the cuticles were incubated with Hyb solution, washed with 50:50 PBT:Hyb solution and PBT, and incubated overnight at 4°C with 1:6000

Roche anti-DIG AP Fab fragments: PBT. Then the next day, the cuticles were washed with PBT and then Staining Buffer, and they were transferred to glass dishes where they were allowed to stain in the dark with Staining Solution, for at least 30min-2hrs (until enough of the pattern showed). They were then washed with Staining Buffer and PBT, and then stored in Glycerol Mountant until they were mounted on slides, also using the Glycerol Mountant. Sense and anti-sense probes for *tan* were used at a concentration of 1:2000, probe:Hyb solution, unless stated otherwise. Protocol taken originally from Sturtevant et al 1993.

Probe preparation: (prepared by A. Neidert) A Bacterial Artificial Chromosome (BAC) plasmid, called 24C15, for exon eight of *tan* was constructed and then cut by EcoRI. The sample then underwent a ligation reaction and PCR amplification to achieve the desired probe for *tan*. The *tan* probe was labeled with DIG using incubation with the PCR template, DIG RNA labeling mix, RNA transcription buffer, DIT, and RNA polymerase. Then the resulting probe was precipitated with linear acrylamide, in order to produce the *tan* probe.

Results

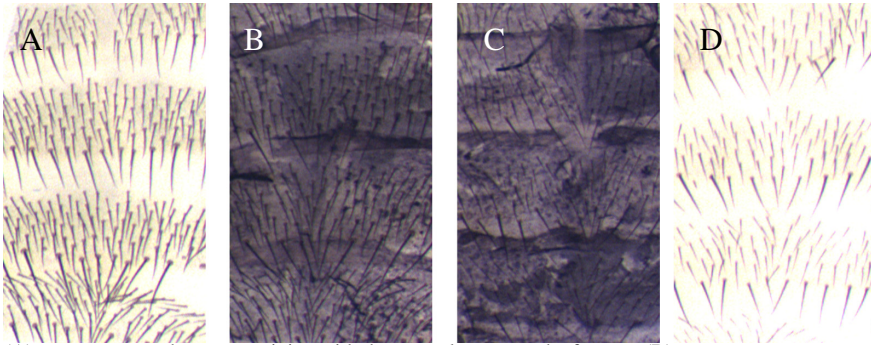
The differences in pigmentation in the thorax and abdomen between *Drosophila americana* and *Drosophila novamexicana* can be seen in Figure 2 from the introduction, which shows the two species at the adult stage, after completed pigmentation. Figure 2A contains an adult from the strain A00, a strain of *Drosophila americana* that has the normal dark pigmentation of *D. americana* throughout the body. The thorax and abdomen are both a dark brown or black color throughout the cuticle, which is different

from the striped pattern normally seen in *Drosophila melanogaster*. Figure 2B shows an adult from the N14 strain, which is a strain of *Drosophila novamexicana*. The thorax and abdomen of this fly are also both pigmented equally throughout the cuticle, again different from the normal striped pattern from *D. melanogaster*. However, unlike *D. americana*, the overall cuticle color of *D. novamexicana* is a much lighter tan or yellow color throughout, instead of the dark brown pigment seen in *D. americana*.

In situ hybridization was used in order to determine the spatial expression of *tan* mRNA in a section of tissue. A probe is prepared in advance, which a labeled strand of RNA (or DNA) complementary to the target mRNA. The cuticles are treated with paraformaldehyde in order to fix the tissue in place and increase access of the probe, and with Proteinase K to open the cell membranes and allow the probe access to the target sequence. Then the probe hybridizes to the target sequence at a higher temperature, and then excess probe is washed off. In this case, the probe was labeled with antigens, so then immunohistochemistry is used in order to localize the labeled bases. The staining solution then allows for the antigen-labeled sequences to develop and show signal, in order to see where the sequence has localized in the tissue.

The in situ hybridization of *tan* RNA at the late stage pupae showed no distinguishable difference in levels of mRNA signal between *Drosophila americana* and *Drosophila novamexicana* at this stage. The abdominal cuticles contained high levels of *tan* mRNA, consistent throughout the entire abdomens of both species, and it was impossible to tell if one species had a stronger signal than the other one. Figure 3 shows the results of the in situ hybridization for *D. americana* and *D. novamexicana*, including the control probes to show that the staining is not background.

Figure 3. Expression of *tan* is constant throughout the abdomen



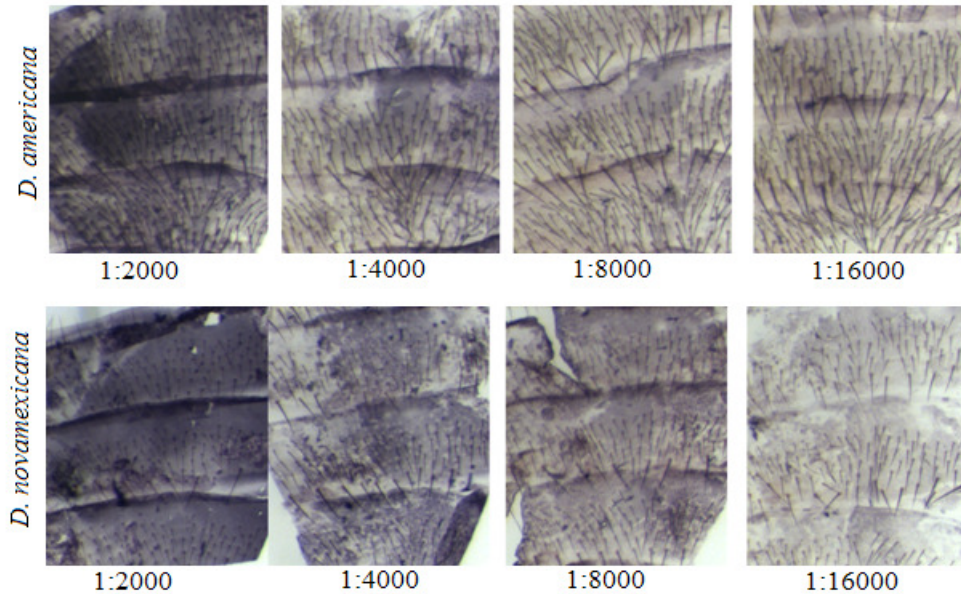
(A) *D. americana* shows no staining with the control, sense probe for *tan*. (B) *D. americana* pupae express high levels of *tan* consistently throughout the abdominal cuticle. (C) *D. novamexicana* pupae express similar levels of *tan* as *D. americana* throughout the entire cuticle. (D) *D. novamexicana* shows no staining with the sense (control) probe for *tan*.

The sense probe, as shown in parts A and D of Figure 3, contains no background signal, suggesting that the *tan* probe is hybridizing to the correct sequence and that there isn't any nonspecific staining of sequences that are not the *tan* gene. The *tan* probe should be antisense to the desired sequence of the *tan* gene and therefore should show signal wherever *tan* mRNA is found. This seems to be the case, since the sense probe should not be able to bind at all, and there is no signal suggesting that *tan* mRNA is present there. Parts B and C contain the antisense probe that should be showing the signal of *tan* mRNA. When compared to parts A and D respectively, these cuticles contain significantly different mRNA expression levels. However, when comparing parts B and C of Figure 3 to each other, there is no noticeable difference in staining levels for *tan* mRNA. The spatial expression patterns for *D. americana* and *D. novamexicana* RNA signal both show constant, high levels of RNA throughout the entire abdominal cuticle. The equal expression of *tan* throughout the cuticle is consistent with the solid pigmentation in *D. americana* and *D. novamexicana*, when compared to the striped pigmentation pattern seen in the abdomens of adult *D. melanogaster* flies. However, the indistinguishable mRNA results between *D. americana* and *D. novamexicana* suggest

that the two species have similar levels of *tan* RNA expression throughout the abdomens of late stage pupae.

In collaboration with Lisa Arnold, I ran an in situ hybridization for A00 and N14, with a dilution series of *tan* probe concentrations of 1:2000, 1:4000, 1:8000, and 1:16000, probe:Hyb solution. In both of the species, the level of expression is very strong, and so one possible reason for the same levels of expression was that the concentrations were too highly saturated to see any subtle difference in mRNA signal between the species. However, we found that, excluding one or two outliers where the staining seemed to be off due to possible cell damage, there still was no discernable difference in mRNA levels (shown in Figure 4).

Figure 4. Concentration Dilution Series for In Situ Staining of *tan* Shows no Differences



A-D show late pupal stage *D. americana* and E-H show late pupal stage *D. novamexicana* with the following concentrations: (A), (E) Concentration of the *tan* probe is 1:2000. (B), (F) Concentration of the *tan* probe is 1:4000. (C), (G) Concentration of the *tan* probe is 1:8000. (D), (H) Concentration of the *tan* probe is 1:16000. No difference in expression is seen when comparing *D. americana* and *D. novamexicana* at the same concentration.

There was a higher degree of variability in levels of mRNA expressed throughout the cuticle at some of the concentrations, probably due to human error by scraping off some of the cells along with fat tissues. However if those outliers are excluded from our consideration, there was no pattern that we could see, and the data seemed to agree with the results from the hybridization study at the high level of concentration. The *tan* signal still seems to be consistent between *D. americana* and *D. novamexicana* at each of the concentrations.

We tried immunohistochemistry using antibodies that should react to Tan protein in parallel with stainings using anti-Ebony antibodies, in order to determine if the antibodies were working correctly. However, the stainings were inconclusive after many repetitions and small variations in procedure, for both Ebony and Tan. We tested Ebony expression along with the Tan protein since we had the antibodies to both proteins, and it would help us determine if we had legitimate staining or only background as Ebony expression had already been determined by Wittkopp et al in 2003. By comparing the results of the antibody stainings to the results using negative controls- the same staining procedure but without the primary antibodies- we were unable to gain any conclusive results. There was no signal from either the Tan or Ebony proteins beyond background noise that was also found in the negative control stainings. Although we knew what Ebony staining should look like, we never saw the correct type of cellular staining we were expecting. Similarly, there was nothing that we could be positive was actual protein staining for Tan either.

Discussion

The highly similar levels of *tan* mRNA expression between *D. americana* and *D. novamexicana* were unexpected, when compared to previous information. As shown by unpublished results from the Wittkopp lab, in the data from the fine scale mapping of *tan*, there is a difference in the pigmentation resulting from the first intron of the *Drosophila americana tan* gene, when compared to the first intron of the *Drosophila novamexicana tan* gene. The hybrids show that when the intron contains mostly the *D. americana* sequence, the hybrid fly has a darker pigmentation in the cuticle. So from this, one would expect that the darker pigmentation of the *D. americana* thorax and abdomen is a result of higher levels of *tan* expression. The mRNA for *tan* is expected to be higher, as is the level of Tan protein expression for *D. americana*. In addition, previous data found that there is a correlation between levels of Ebony expression and the melanin differences of *D. americana* and *D. novamexicana*, but there was no such correlation between pigmentation and Yellow (Wittkopp et al 2002). This explains why *D. novamexicana* has a more yellowish tint to the cuticle, but it does not explain the darker pigmentation of *D. americana*. Yellow and Tan are the two proteins that lead to darker melanin in the pigmentation pathway, and yet from the results that Wittkopp et al (2002) found and the above data, it seems that neither *yellow* nor *tan* genes have stronger mRNA signals in *D. americana*. This then leads to the question of whether Tan levels of expression are higher at some stage other than the late pupal stage being studied in the in situ hybridization.

One possible reason for the similar mRNA signals in *D. americana* and *D. novamexicana* was that the concentrations of *tan* probe in the first set of in situ results

(Figure 3) might be oversaturated, preventing us from detecting any difference in signal levels. However, by studying the cuticle stainings in the concentration dilution series (Figure 4) and not ignoring any extreme outliers we saw, we found no pattern showing a difference in mRNA levels between the species at any concentration. Therefore, the high concentration of the *tan* probe didn't seem to be causing an oversaturation of mRNA that would explain the similar expression patterns between the two species. Instead, it seems that at this stage, both *D. americana* and *D. novamexicana* have highly similar mRNA signals for *tan*.

It is also possible that although the mRNA levels of *tan* seemed equal, there could still be a difference in levels of Tan protein expression between *D. novamexicana* and *D. americana*. However, we were unable to conclude anything about the levels of Tan expression in *D. americana* and *D. novamexicana* through immunohistochemistry, and we don't know if the Tan expression levels show any correlation to the pigmentation differences, as is expected. This is something that will need to be tested in the future with a working antibody.

Another possible explanation for the unexpectedly similar *tan* mRNA signal is that while the *tan* mRNA levels seem to be equal at this stage, the signals might diverge at a different point in the development of the flies. The in situ hybridizations were conducted with late stage A00 and N14 pupae, which occurs after the wings have turned black and before eclosion of the pupae into adults. Previous studies involving *tan* expression levels have also studied the flies at the same late pupal stage, shortly before eclosion (Wittkopp 2002; Jeong 2008). However, in their study showing the difference between Ebony expression levels of *D. americana* and *D. novamexicana*, Wittkopp et al

(2003) studied adult cuticles instead of cuticles from the late pupal stage. Therefore it is possible that the adult cuticles of *Drosophila americana* and *Drosophila novamexicana* might show differences of *tan* mRNA, as was found for Ebony protein levels at that stage. It is also possible that Tan might be expressed differentially earlier in development and that by the late pupal stages the levels of protein expression have become more even. In order to determine if *D. americana* and *D. novamexicana* have different expression levels of Tan at a different developmental stage, it is necessary to study the levels of Tan protein and *tan* mRNA at the different pupal and adult stages of the flies.

Developmental Time Series of *D. americana* and *D. novamexicana*

Although it is known that *D. americana* and *D. novamexicana* take longer to develop than *D. melanogaster* during morphogenesis, the exact time series of the two species has never been studied. The approximate turnover rate can be seen through weekly fly transfers. From this, *D. melanogaster* seems to take around two weeks for a reproductive cycle, while *D. americana* and *D. novamexicana* take closer to one month. However, there has not been any study yet determining the precise differences in developmental rates among those species. From previous work first conducted by Bainbridge and Bownes (1981), the developmental process of *D. melanogaster* has been broken up into well-known stages, but there has yet to be a comparison between the times these stages take for the different *Drosophila* species. Therefore, before studying how the *tan* gene might be expressed differently at different stages, it is first important to analyze the morphology of the species, compared to *D. melanogaster*, throughout the life cycle.

In their paper from 1981, Bainbridge and Bownes map out a staging series for the development of *Drosophila melanogaster* from the embryonic stage until the fully developed adult. From this, they found a sequence of 51 noticeable changes in events that take place throughout the morphogenesis by analyzing the imaginal disks to study the development (Bainbridge and Bownes 1981). This paper was the first one to develop a method of studying staging the pupae, and it also provided the basis for future developmental staging studies, along with information about staging used in any *Drosophila* manual or textbook. Using the wild type Ore-R *D. melanogaster* stock, incubated at 25° C, they followed the flies throughout the different stages and calculated

the various times for each stage (Bainbridge and Bownes 1981). Bainbridge and Bownes (1981) found that the events became less synchronous as time went on, and there was more variability in the times for each stage as this occurred. The duration of each stage decreased as the flies progressed, so the increased variability is probably related to the shorter duration time of the stages. They repeated the staging experiment using *D. simulans* and *D. pseudoobscura* and found similar results for the basic stages involved in pupal development and the morphological changes associated with each; though they did not conduct the developmental study for any flies in the *D. virilis* subgroup. The 52 different events they found were later used as tools for Michael Ashburner in his book, *Drosophila: A Laboratory Manual*.

In his manual, Ashburner (1989) outlined the stages of pupal and pharate adult development, based on previous staging studies. From these studies, he provides the details on how to recognize each stage of larval, pupal, and adult development and the approximate time it takes for a female and male *D. melanogaster* in each stage. The stages that were particularly useful for the developmental staging of *D. americana* and *D. novamexicana* were starting at Stage P8 of pupal development, where morphology is easily recognized. Ashburner describes the stages from that point until the fully developed adult as follows: P8 is characterized by yellow eyes; P9 flies have amber eyes; P10 flies have red eyes and are bald; P11 flies gain head and then thoracic bristles; P12 flies have grey wings or wing tips; at P13 the wings turn black; at P14 the mature bristles appear and the green meconium appears at the posterior tip; P15 pupae are tan so that the tubules and yellow body are obscured; at A1 eclosion occurs and the wings are folded; at A2 the wings extend and the abdominal cuticle begins to darken and gain pigmentation;

and finally the A3 flies are fully pigmented, fully developed adults (Ashburner 1989). The A2 and A3 flies are very similar morphologically as they both involve adult flies with the wings extended, so in this study they were combined into one inclusive stage.

Ashburner also describes the approximate time for each stage, for males and females, although both can vary for each individual fly. According to his book and the study conducted by Bainbridge and Bownes, the average total duration of development for *D. melanogaster* is approximately 113 hours for females and 118 hours for males, while the total metamorphosis time- from the larvae up until eclosion- is around 98 hours for females and 102 hours for males (Bainbridge and Bownes 1981; Ashburner 1989). The average total developmental time, according to Bainbridge and Bownes, is around 115 hours for a *D. melanogaster* fly, from the larval stage until the fly is a fully developed adult, although there is variation for each individual (Bainbridge and Bownes 1981). The earlier stages generally take longer- P8 and P9 are both around 12 hours, while there is only a two hour duration for P15, before the fly ecloses (Bainbridge and Bownes 1981). The later stages are also more similar looking, since P8, P9, and P10 all have different color eyes but then the rest all have red eyes; and stages P13-15 all have dark wings. The shorter duration time then makes sense because less time is necessary for smaller changes.

Although these studies provide the basis for staging a *D. melanogaster* fly, there has been no previous literature that describes a developmental time series for either *D. americana* or *D. novamexicana*. Since the generation time for *D. melanogaster* is approximately two weeks while that of *D. novamexicana* or *D. americana* is four to six, it is expected that those two species will age much more slowly than *D. melanogaster*, and

that there will be different times for each stage of development. *D. americana* and *D. novamexicana* seem to have similar generation times, so I expected that there isn't a large difference in the staging for each species, but as the developmental series hadn't been studied before, I thought it might be interesting to find out and make sure. The developmental staging for *D. melanogaster* was useful in further studies involving the temporal expression of genes and proteins. So staging the development of *D. americana* and *D. novamexicana* should prove useful for studying the spatial and temporal expression of Tan in the two species.

I will show that after analyzing the developmental time series, *D. melanogaster* was proven to age at a faster rate than either *D. americana* or *D. novamexicana*. In addition, I will show that there is no significant difference in the rate of development for *D. americana* and *D. novamexicana*, especially when comparing the slight difference in developmental rate to the amount of variability seen in the duration of each stage.

Materials and Methods

Drosophila strains: The three strains that were used were *Canton S* for the wild type *D. melanogaster*, A00 for wild type *D. americana*, and N14 for wild type *D. novamexicana*. All three were raised on standard cornmeal media at room temperature until the staging process.

Aging: 60 wandering third instar larvae of each strain were taken out of their vials and distributed into new vials with the same type of media, with 20 larvae of one strain per vial. These vials were incubated at 20° C until pupal shells had formed and the pupae had hardened. Then the pupae were moved onto pieces of tape (sticky side facing up),

one for each species, which were attached to plastic boards. These were put in clear boxes in order to prevent the flies from escaping once they became adults. The boxes were placed back in the incubator at 20° C and the flies were allowed to age naturally, until the experiment was complete and all flies had developed into adults.

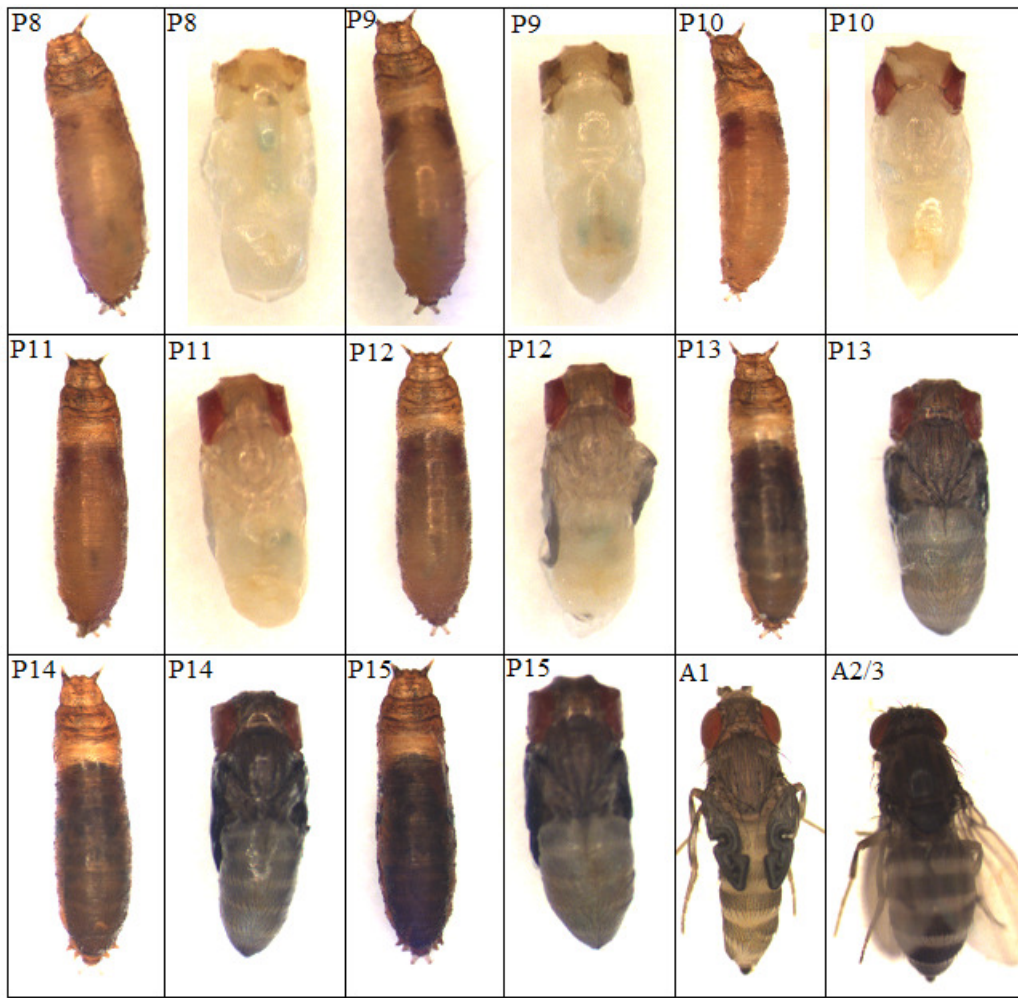
Determining the duration of each stage: The boxes were checked every two to six hours, and each time a random sample of five flies from each species was analyzed. These flies were checked under a microscope, both in the shell and outside of it if necessary, in order to determine the stage each was at, starting once the eyes turned yellow. The stages of those five flies for each strain were recorded at that time.

Photography: A00 and N14 flies were photographed both inside and outside of the pupal shell under the microscope at each stage, using 12x magnification each time. They were photographed using a Leica MZ6 microscope and a Scion color digital camera, model CFW-1308C.

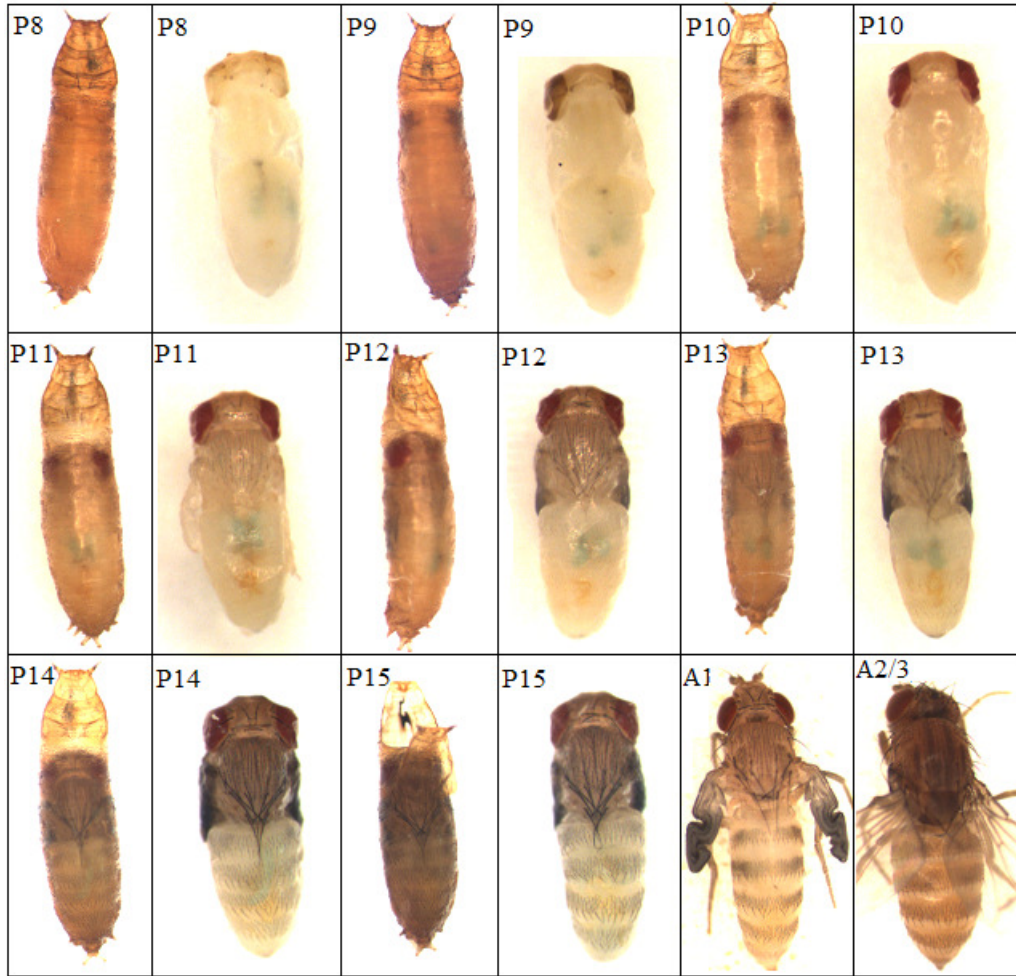
Results

The developmental stages were recorded and analyzed for each of *Drosophila americana*, *Drosophila novamexicana*, and *Drosophila melanogaster*. *D. melanogaster* finishes developing much earlier than *D. americana* and *D. novamexicana*, but there doesn't seem to be a difference between the developmental rate of *D. americana* and *D. novamexicana*. Figure 5 depicts each stage of development for *D. americana* and *D. novamexicana* while Figure 6 shows a linear regression model for the stages that each of the five sample flies is currently showing at the different times they were looked at, for each of the three species.

Figure 5. Stages P8-A2/A3 of N14 and A00, with and without pupal cases



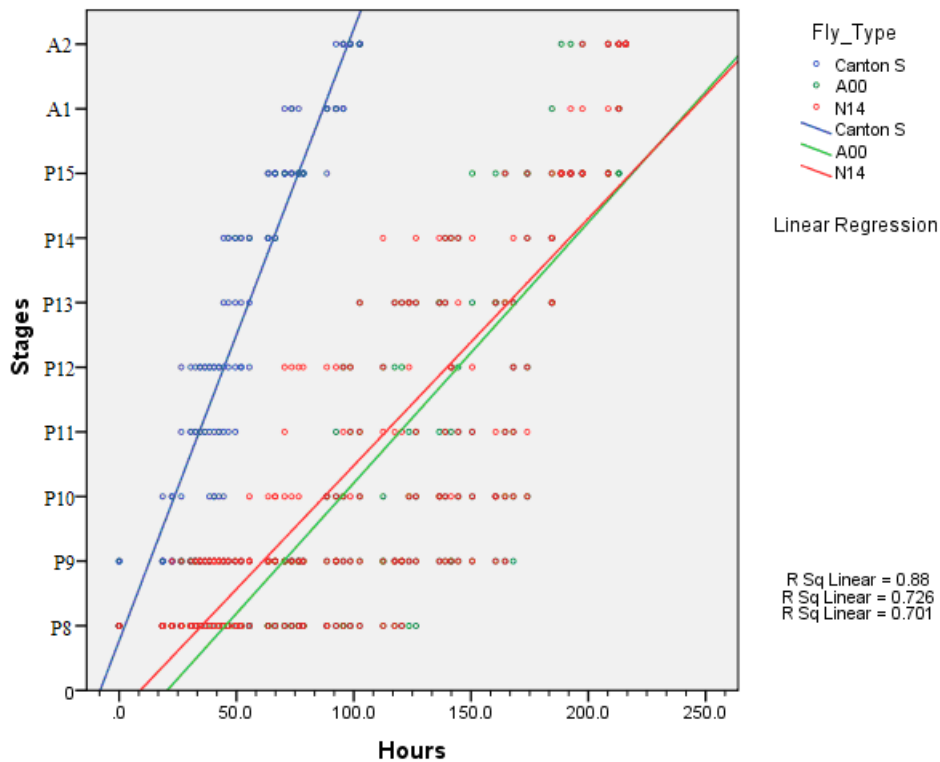
A.



B.

(A) *D. americana* stages are shown with the pupae inside and then outside of the pupal shell. (B) *D. novamexicana* stages are shown with the pupae inside and then outside of the pupal shell. There seems to be no difference in the morphological development of *D. americana* and *D. novamexicana*, and the same physical markers can be seen in each stage.

Figure 6. Relationship between time and stages of development



The number of flies out of a five-fly sample at each stage is calculated over time for *D. americana*, *D. novamexicana*, and *D. melanogaster*, labeled as A00, N14, and Canton S, respectively.

Figure 6 shows the different stages of the flies and how many flies are at each stage, at different times throughout the development. The graph shows R-squared linear regression value for each species, consisting of 0.88 for *Canton S*, 0.726 for A00, and 0.701 for N14. In addition, *Canton S*, which again is the *D. melanogaster* strain, ages more quickly than either *D. americana* or *D. novamexicana*. There is also less variability with respect to the stages each fly is in for the *D. melanogaster* strain. The linear regression lines show a projected slope, which represents the rate of development. Since the R-squared value for the *D. melanogaster* strain is significantly higher than either of the other two, this means that more of the data points lie closely to the line and that the

line is a better fit for *D. melanogaster*. The graphs for all three strains are significant since the R-squared value for all three is over 0.7, so there is a strong correlation with the data points and the given regression line. However, there is definitely more variation in the staging of *D. americana* or *D. novamexicana* since a smaller percentage of the data points for those two are represented by the developmental slope predicted by the regression line. There is also no significant difference in the staging timeline of either of those two species, which was more or less expected since the turnover rate is so similar.

Discussion

There is still a clear difference between the time it took for *D. melanogaster* to age and the time it took for the other two species, but they are closer than one might expect. Part of this is probably due to the temperature used for incubation in this developmental study. Bainbridge and Bownes (1981) grew and aged *D. melanogaster* flies at 25° C, which is an optimal temperature for *D. melanogaster*. However, this temperature is warmer than the optimal one for incubation of *D. americana* or *D. novamexicana*. In this study, the flies were kept in an incubator that was set at 20° C. This was a more hospitable environment for *D. americana* and *D. novamexicana*, which are both harder to grow and sustain than the more resilient wild type *D. melanogaster*. Therefore, the colder temperature slowed down the developmental rate of *D. melanogaster*, thus causing the closer similarity between the three species than what was expected. It's also possible that the other stages before the one I started with might take much longer for *D. americana* and *D. novamexicana*, and so to test this I would want to see how long it takes for each species to mature to the P8 stage after being eggs. In

addition, it seems to take longer for *D. americana* and *D. novamexicana* virgin flies to mate and then lay eggs. So there might be a longer generation time because it takes longer to lay new eggs. This can also be tested in the future by placing virgins and males of each species in a vial and seeing how long it takes for there to be larvae inside each vial. However, even though the times were closer than expected, it is still clear that *D. melanogaster* ages at a faster rate than *D. americana* or *D. novamexicana*. The size of the flies also makes a difference in developmental time. *D. melanogaster* is noticeably smaller in body size than *D. americana* and *D. novamexicana*, which are approximately the same size. Therefore it takes longer for *D. americana* and *D. novamexicana* to develop, since their bodies are larger.

D. americana and *D. novamexicana* show no significant differences in the developmental time series. Both species seem to age at similar rates, when taking into account the amounts of variability. This does not deviate from the expected results, since the two species reproduce at similar rates in the laboratory. In addition, the species probably encounter a larger degree of variability due to the slower rates of development, when compared to *D. melanogaster*. The larvae were collected when they were wandering around in the vial, but it is possible that this stage lasts a long time as well. Since *D. americana* and *D. novamexicana* age more slowly, there was a larger variability in ages to start the experiment, and so it make sense that they would be less consistent throughout the developmental process.

The staging process, while failing to show more morphological or behavioral differences between *D. americana* and *D. novamexicana*, is useful for future studies involving the development of gene or protein expression. Mapping out the

developmental stages has given a good idea of when the pigmentation starts to be visible. Figure 5 suggests that pigmentation first shows up around stages P12 or P13 in both *D. americana* and *D. novamexicana*. This information can be helpful when comparing the developmental progress of genes to the physical development of the flies. In particular, I mentioned earlier that *tan* seems to be expressed equally at the late pupal stage- now identified as either stage P14 or P15. However, it's possible that sometime earlier or later in the aging process, the levels of RNA or protein expression corresponding to the *tan* gene differ between the two species. With the knowledge gained from this staging project, it is easier to classify the age of the pupae. In addition, each individual ages at slightly different rates. It is harder, therefore, to classify each individual by time, since there can be more than one stage at one particular time. By referring to the stage instead, we can have more consistent results because the individual pupae or adult flies will actually be closer in age this way. Therefore the time series is useful for future studies involving pupae or larvae.

Now that the time series has been studied, it is possible to see if the levels of Tan protein are consistent between *D. americana* and *D. novamexicana* throughout the development of each species. The two species age in parallel so it will be easy to analyze the RNA or protein expression at varying stages of development, in order to see if there is any divergence of expression levels or if Tan is always equal in its expression. Therefore, from the data gained from the developmental time series of *D. melanogaster*, *D. americana*, and *D. novamexicana*, along with the results of in situ hybridizations and other tests at the late pupal stage, it is possible to see if Tan expression differs between

the two species at any point in their development, between stages P8- yellow eyes- and A3- a fully developed adult.

Developmental Expression of Tan in *D. americana* and *D. novamexicana*

From earlier in this paper, it was determined that *D. americana* and *D. novamexicana* have similar levels of *tan* mRNA expression at the late pupal stage- stage P14 or P15. In addition, Western blots completed by Lisa Arnold (unpublished data) have shown similar levels of Tan expression at that stage. However, it is possible that at some point in the development of the flies, the expression levels of Tan change and one becomes stronger than the other.

In a similar study conducted by Walter et al (1991), the temporal and spatial expression of *yellow* was studied, in correlation with the development of the *Drosophila* cuticle. In this study, they studied the expression levels of *yellow* throughout the larval and pupal development, through the full development into an adult. They found that the *yellow* gene has two activity peaks throughout the development of the fly: one occurring during the late embryonic development and the other one occurring in the second half of pupal development (Walter et al 1991). Using immunohistochemistry to study the temporal and spatial expression patterns of Yellow, they found that pigmentation followed an anterior to posterior pattern of development, from the bristles to the thorax, to the body; and they also saw that the pattern of melanin deposition was also in the in the bristles of the head and thorax until eclosion (Walter et al 1991). Since the Yellow protein can be present and functioning before pigmentation development Walter et al (1991) hypothesize that the deposition of *yellow* also follows the same anterior to posterior pattern, though they have yet to prove this developmental spatial pattern. Through the staining procedure, they found positive staining with the anti-yellow serum in pupae at least 48 hrs old and localization in the epidermal cells starting at 58 hrs and

continuing on throughout development (Walter et al 1991). They also showed that the earliest detection of the *yellow* gene occurred at the same time as the beginning of adult cuticle secretion (Walter et al 1991). Thus, they suggest that there is a correlation between the expression of Yellow and cuticle secretion in *Drosophila*. Using a developmental staging of Yellow, Walter et al (1991) were able to analyze the function of the protein in *Drosophila* and show a correlation to cuticle secretion, which they would not have been able to do as successfully without analyzing the temporal expression of Yellow.

This paper gives a method for analyzing the development of a pigmentation gene and its proteins, corresponding to the development of the flies. The paper provides a basis for my work, where I have studied the developmental expression of the Tan protein in *D. americana* and *D. novamexicana*, using *D. melanogaster* (wild type strain *Canton S*) in parallel as a sort of control. I hypothesize that the expression of Tan occurs slightly differently for *D. americana* and *D. novamexicana*, despite the similar levels of expression at the late pupal stage, since this would help explain the difference in abdominal cuticle color. I also hypothesize that Tan will show the strongest signal towards the end of development, when pigmentation shows up and when Ebony has been shown to start being expressed. However, I will show that the preliminary results, which will have to be repeated at a further time for validation, show the opposite of what was expected and that Tan signal shows up the strongest earlier in the pupal development of *D. americana* and *D. novamexicana* while it shows up the strongest later, right before eclosion, in *D. melanogaster*.

Materials and Methods

Drosophila strains: The three strains that were used were *Canton S* for the wild type *D. melanogaster*, A00 for wild type *D. americana*, and N14 for wild type *D. novamexicana*. All three were raised on standard cornmeal media. A00 and N14 were aged at 20°C and *Canton S*, at 25°C until samples were collected.

Protein Extraction/Collection: Five whole bodies, five heads only, and five bodies only (no heads) were collected for each of *D. melanogaster*, *D. americana*, and *D. novamexicana* at the following stages: P8, P9, P10/11 (red eyes), P12, P13-15 (black wings), A1, and A2/3 (fully extended wings, complete pigmentation). These were collected over a period of time and stored, without being squashed in the buffer, at -80°C until all samples were ready for extraction. Then all of the protein samples were extracted the same day. When the protein was extracted, the flies were squished in Homogenizing Buffer and then centrifuged at room temperature. The supernatant was transferred into new vials along with an equal volume of Sample Buffer and boiled for ten minutes. Then the samples were aliquotted into 25µl samples and stored at -80°C.

Western Blots: Due to lack of time and possible significance, Western blots did not include *D. americana* or *D. novamexicana* stages with the heads only. However, all of the other stages were tested with the whole fly and the body (thorax and abdomen) only for *D. americana* and *D. novamexicana*; and *D. melanogaster* was tested with all stages and all three categories of body parts. 15% polyacrylamide resolving gels were made with 4% polyacrylamide stacking gels on top. The samples were loaded into the wells and the gel ran for around 75 min at 130V. The gel was transferred onto PVDF membrane after being in a blotting buffer and running for 60min at 350mAmps. The

membranes were washed with TBST and blocked with milk for 2 hours. Then they were washed again with TBST and allowed to incubate overnight at 4°C with the primary antibody (Rabbit anti-actin (1:4000) and either Rabbit anti-tan pur 642 (1:10000) for *D. americana* and *D. novamexicana* or Rabbit anti-tan ap 63 (1:1000) for *D. melanogaster*). Then the membranes were washed with TBST the next day, incubated at room temperature with secondary antibody (anti rabbit peroxidase, 1:15000) for two hours, washed again with TBST and TBS, and covered with developer and then allowed to develop for varying amounts of time onto film. This procedure is adapted from Sambrook and Russell 2001.

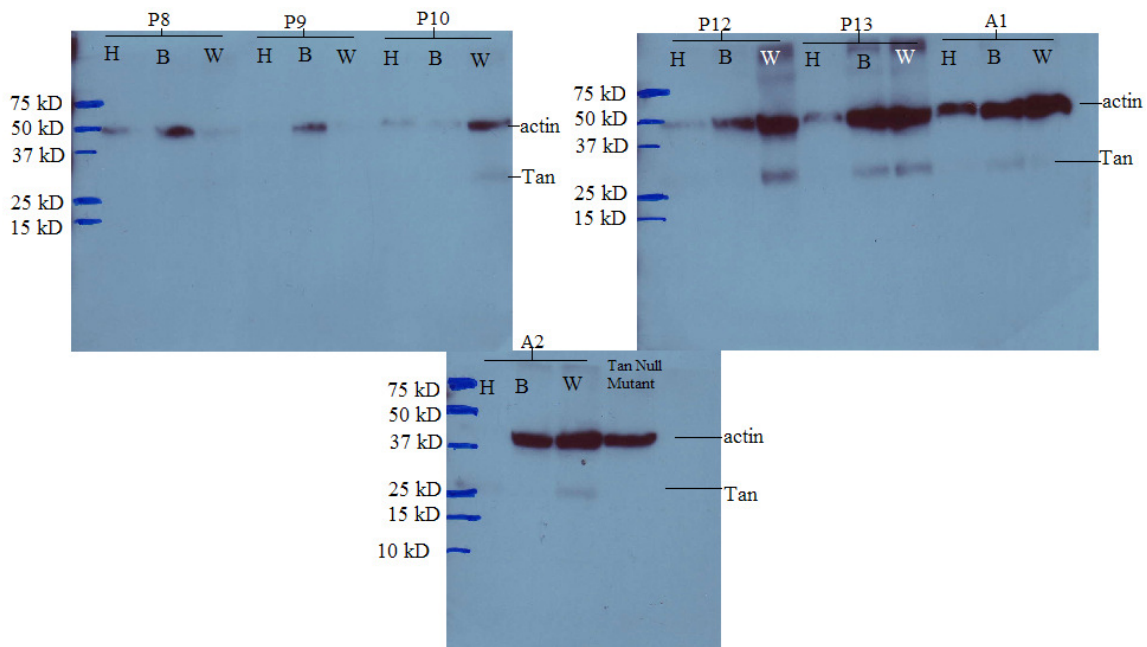
Results

Western blots are used to detect specific proteins in a sample of tissue based on the size, and so they were used in this study in order to determine the level of protein expression for different stages of *Drosophila* species. Gel electrophoresis is used in order to sort proteins and separate them according to size, since smaller proteins move more quickly than larger ones. The proteins are then transferred onto a membrane, and they are probed using antibodies specific to the target protein. In this study, the membranes were co-stained with antibodies for Tan and anti-actin antibodies, in order to set up a loading control. A secondary antibody is used to react to both of the primary antibodies, and this one is a peroxidase that produces luminescence in proportion to the amount of protein. The membranes show the presence of Tan in each stain by showing the relative strength of the band corresponding to the size of the *tan* gene. The membranes then are

developed with film paper, and the luminescence from the protein levels cause light exposure onto the film, creating an image of the levels of Tan protein at each stage.

Drosophila melanogaster showed the expected pattern of expression of Tan, peaking at the late pupal stage, P13. The Western blot for the *D. melanogaster* time series is shown below, in Figure 7. Bands representing actin- the loading control- and the *D. melanogaster* Tan are labeled as such.

Figure 7. Developmental expression of Tan in *Drosophila melanogaster*



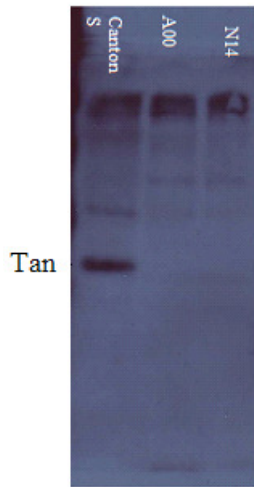
Western Blots showing the expression of Tan for developmental stages in *D. melanogaster*. H represents the heads only, B represents the bodies only, and W represents the whole flies.

The gels for *D. melanogaster* show that Tan expression starts at low levels in the whole fly at stage P10 in the pupal development, and the strongest expression of Tan occurs for both the whole fly and the body only at stage P13, right before eclosion. The actin bands for body only and whole fly are stronger than the ones for stages with only the head, since there is more protein being loaded in the well. In addition, there is a

stronger band for actin for most of the whole fly wells when compared to the body only ones for the same reason. The first gel shows consistently lower levels of actin staining, and the bands for actin at P8 and P9 for the whole fly are also weaker. This is an indication that the amount of protein in those wells was not as high as they levels in other wells. So the lower level of actin shows that the loading of protein in the wells is not quite equal. Therefore it is hard to compare the level of band intensity of Tan in the gel with low intensity actin staining with one with high levels of actin. Nonetheless, the gels show the predicted pattern based on previous knowledge of other genes in the pigmentation pathway. As mentioned above, the Yellow protein peaks at a late pupal stage, and here, too, Tan shows the same peak at the last pupal stage before eclosion. The results shown in Figure 7 support the predicted model for temporal expression of Tan.

Once the developmental pattern of expression for Tan in *D. melanogaster* was determined, I tested how the expression patterns looked for *D. americana* and *D. novamexicana*. However, the antibody used for the *D. melanogaster* Tan protein did not work for *D. americana* and *D. novamexicana*. Previous work by A. Neidert (unpublished data) shows that the Tan antibody used for *D. melanogaster*, Rabbit anti-tan ap 63, does not cross react with *D. americana* and *D. novamexicana* (shown in Figure 8).

Figure 8. The *D. melanogaster* Tan antibody does not cross react with *D. americana* or *D. novamexicana*



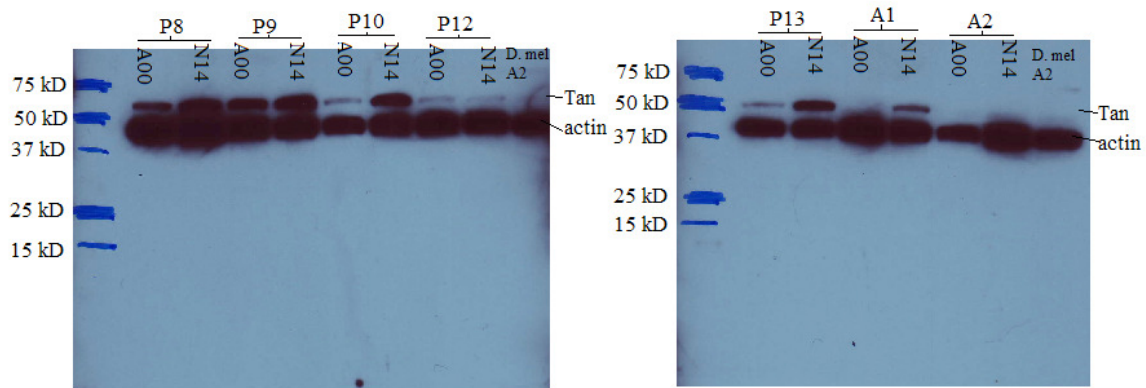
Expression of Tan in *D. melanogaster* (Canton S), *D. americana* (A00), and *D. novamexicana* (N14), using the *D. melanogaster* Tan antibody shows that *D. americana* and *D. novamexicana* don't react with that antibody.

The gel shows the band for Tan in the *Canton S* strain, which represents *Drosophila melanogaster*. However this band does not appear in the *D. americana* strain (A00), the *D. novamexicana* strain (N14), or any of the transgenic lines where the *D. americana* and *D. novamexicana tan* genes were inserted into a *D. melanogaster* background. So the antibody for Tan in *D. melanogaster* does not seem to cross react with Tan in *D. americana* or *D. novamexicana*. There will have to be some other antibody to use for those two species then, in order to test their expression of Tan.

We formed an antibody for Tan that seemed to work for *D. novamexicana* and *D. americana*, and after it was tested by A. Neidert (unpublished data), we assumed that the antibody was correctly binding to Tan in *D. americana* and *D. novamexicana*. The new Tan antibody, Tan pur. 642, showed bands at around the 50 kD region in Western blots for *D. americana* and *D. novamexicana*. It has been proposed that this is the approximate length of *tan*, so the antibody seemed to be the correct one. I used this antibody for the

developmental series of Tan expression in *D. americana* and *D. novamexicana*. Figure 9 shows the results for the thorax and abdomen regions only, and no heads, of each species at the different stages.

Figure 9. Developmental Expression of Tan in *D. americana* and *D. novamexicana* bodies only



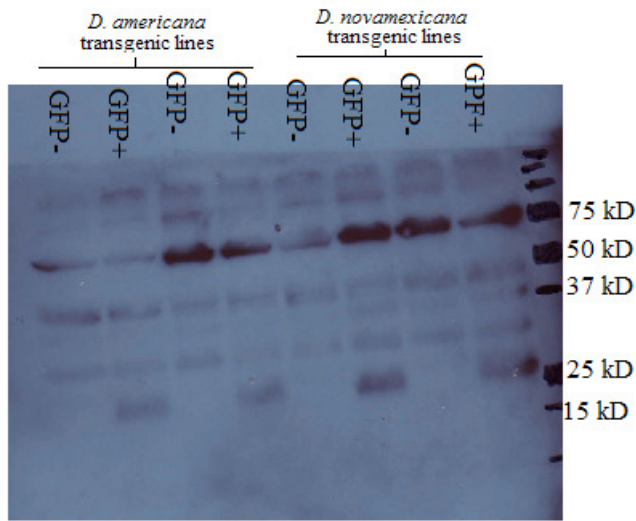
Expression of Tan is shown for *D. americana* (A00) and *D. novamexicana* (N14) through different stages of development, using protein from the bodies only.

Again, A00 is the strain representing *D. americana* while N14 represents *D. novamexicana*. From this image, it appears that Tan expression in the bodies (without the heads) of *D. americana* and *D. novamexicana* starts at the earliest stage in the gels, at P8 in the pupal development. Then around stage A10, when the pupae gain red eyes, the level is reduced for *D. americana*, though it's hard to compare the level of Tan in *D. americana* and *D. novamexicana* at this stage, because the actin level for *D. americana* is also lower than that of *D. novamexicana*. At stage P13, where the peak of Tan expression is shown in *D. melanogaster*, *D. americana* has lower levels of Tan, while *D. novamexicana* seems to have a much higher level of Tan expression than *D. americana*. The actin levels of both species at that stage seem equal, so this suggests that there wasn't an error in loading the protein into the wells. Instead, it appears that Tan protein

expression is higher for *D. novamexicana*. This is surprising and unexpected, because as described earlier, Tan leads to dark brown melanin formation. *D. americana* has a much darker thorax and abdomen than *D. novamexicana*, so based on their pigmentation differences, Tan was expected to be expressed more highly in *D. americana*, not *D. novamexicana*.

In *D. melanogaster* there was no Tan expression in stages P8 and P9, while the protein levels are shown to be the strongest for *D. americana* and *D. novamexicana* at those stages. In addition, transgenic lines were created by L. Arnold (unpublished data). In this flies, *D. americana* or *D. novamexicana tan* was inserted into a *D. melanogaster* background, so that only *tan* from the *D. americana* or *D. novamexicana* was expressed. The flies were shown by L. Arnold et al (unpublished) to retain the *D. americana* or *D. novamexicana* pigmentation and not the phenotype of *D. melanogaster*, suggesting that the *tan* from those species was in fact turned on in *D. melanogaster*. The inserted *tan* gene was linked with Green Fluorescent Protein (GFP) in order to have a way to see that the correct *tan* is being expressed. So GFP is used as a marker to show whether the sequence was injected into *D. melanogaster* or not. Since GFP is linked to the foreign sequence, whenever the transgenic fly is GFP+, it should be expressing the *tan* sequence from either *D. americana* or *D. novamexicana*. The *D. americana* and *D. novamexicana* transgenic lines were tested with the same Tan pur. 642 antibody (unpublished data by L. Arnold). The results of this Western blot are shown below in Figure 10.

Figure 10. Tan expression is not seen in *D. americana* or *D. novamexicana* transgenic lines at the expected band size



Transgenic lines for *D. americana* and *D. novamexicana* show a signal at 25 kD in the GFP+ flies while the GFP- flies show no signal at that level. No such pattern is seen for bands at around 50 kD.

As mentioned earlier, the *D. americana* and *D. novamexicana* Tan protein should be found at a level of around 50 kD. In the gel in Figure 10, the only wells that should express the Tan protein would be the ones that are GFP+, because those would be expressing *D. americana* or *D. novamexicana* Tan instead of Tan from *D. melanogaster*. However, the only level of protein expression where there is a correlation with GFP is at 25kD, which is much smaller than the Tan should be but the size of the cleaved Tan in *D. melanogaster*. So it is possible that the Tan protein is cleaved in the transgenic flies as well, since this is approximately the size of Tan in *D. melanogaster*. It is also possible that the transgenic lines of inserted *tan* sequence in a *D. melanogaster* background don't express Tan with this antibody, but this is not as likely because we know that Tan is expressed from results showing that the *D. americana* or *D. novamexicana* phenotype is rescued in a *tan* mutant *D. melanogaster* background.

Discussion

The Western blots showing expression of Tan in *D. melanogaster* have a pattern that is expected, knowing how other pigmentation genes are expressed throughout development. However, the developmental expression series for Tan in *D. americana* and *D. novamexicana* show the exact opposite results. In the results from those Western blots, Tan is shown to be expressed highly in the earlier stages of pupal development, and then at an intermediate stage- P10- the protein is down-regulated. From the study by Wittkopp et al (2002), we know that Ebony is expressed at later pupal stages, and True et al (2005) showed that Tan only functions after Ebony biochemically, due to the reciprocal functions of the two proteins. Therefore, if Ebony is expressed in later stages and not in the early stages, it does not make sense that Tan would be turned on in the earlier pupal stages and not show expression in the later stages.

If the results from the Western blots of *D. americana* and *D. novamexicana* Tan expression throughout development are accurate, then Tan is expressed much earlier in development than we expected. From True et al (2005), we know that Tan has more than one function in the fly, and that it is important in the pigmentation pathway and for neural systems in the eyes. It is possible that Tan also plays a function in other neural systems in the body of the fly, and that this pathway might function much earlier in development than the pigmentation pathway. One way to help test this hypothesis would be to run the same developmental Western series on the Ebony protein levels in *D. americana* and *D. novamexicana*. In both of Tan's functions, there is still a reciprocal part of the pathway between Tan and Ebony. Therefore, it can be assumed that if Tan is being used earlier in development, Ebony is being expressed at those stages as well. Therefore, if Ebony

expression levels were also present at stages P8 and P9 in pupal development, this could help validate the results from the Westerns.

One other unexpected result from the Western blots for *D. americana* and *D. novamexicana* is that from the P10 stage through the rest of the pupal development, *D. novamexicana* appears to be expressing a higher level of Tan protein in the body than *D. americana*. As mentioned earlier, *D. novamexicana* has a much lighter cuticle color than *D. americana*, and since Tan leads to dark pigmentation, one would expect *D. americana* to produce the higher levels of Tan. However, one possible explanation is that the levels of Tan and Ebony are not the important deciding factors alone for determining melanin, but instead the ratio of Tan to Ebony expression is important. *D. novamexicana* expresses higher levels of Ebony than *D. americana*, so it's possible that it can also express higher levels of Tan, but that the ratio of Ebony:Tan in *D. novamexicana* is higher than that of *D. americana*. This would be more difficult to test, because the antibody for Ebony is different from the one for Tan. However, it is possible to biochemically test the flux rates of dopamine to NBAD and NBAD to dopamine, in order to see if there's a higher rate of conversion from NBAD to dopamine in *D. americana*. If this is true, then it would suggest that the expression of Tan is higher than that of Ebony in *D. americana* while the opposite would be expected for *D. novamexicana*.

However, while these possible explanations could show why the results are so surprising, another possibility is that the antibody for *D. americana* and *D. novamexicana* is not actually binding to Tan but instead to a protein of similar size. The Western blot from A. Neidert (unpublished; shown in Figure 8) shows that the Tan antibody used for *D. melanogaster* does not also work for *D. americana* or *D. novamexicana*, so members

of the Wittkopp lab had to create a new antibody to use instead. It appeared that the tan pur. 642 antibody, used in this experiment for *D. americana* and *D. novamexicana*, bound to Tan protein in those species. However, this does not prove that it recognizes Tan. The ways to test whether an antibody is the correct one and binds to the desired sequence are to test it against a null mutant or test it with an over-expressed protein. There is no tan null mutant for *D. americana* and *D. novamexicana*, however, so it was impossible to test the antibody against a null mutant fly. Likewise, there aren't really any flies with over-expressed Tan from *D. americana* or *D. novamexicana*. The closest available flies to use instead are the transgenic lines of *D. americana* or *D. novamexicana* tan sequence inserted into *D. melanogaster*.

The transgenic flies used in the Western by L. Arnold contain sequences from those flies injected into the *D. melanogaster* background. So they should behave similarly to native *D. americana* and *D. novamexicana* flies in the expression and binding patterns of Tan. However, the only band expressed by GFP+ flies and not GFP- ones in that gel is a much smaller size than that of Tan. Tan was shown to be cleaved in *D. melanogaster* (Wagner et al 2007), but it doesn't appear to be cleaved in *D. americana* or *D. novamexicana*, which explains the shorter band size for *D. melanogaster* Tan when compared to the size of Tan in *D. americana* and *D. novamexicana*. It is likely that Tan is cleaved in the transgenic flies as well, as it is in *D. melanogaster*, but then the smaller band size is not seen in *D. americana* or *D. novamexicana* flies so it does not appear to be cleaved there. So there doesn't seem to be any evidence of a Tan protein the same size as the Tan protein seen in *D. americana* and *D. novamexicana* being expressed in the GFP+ flies and not the GFP- transgenic flies, according to the Western blot in Figure 10.

Therefore, due to the lack of definitive evidence proving that the antibody is actually binding to Tan in *D. americana* and *D. novamexicana*, it is possible that the Western blots in Figure 9 are not actually showing Tan expression levels. The results in Figure 7 show very a very different development of Tan expression than the proposed temporal expression as seen in Figure 9. In addition, there has been no definitive proof as of yet that the antibody for Tan in *D. americana* and *D. novamexicana* is in fact the correct antibody. It is just as likely that the antibody is binding to a protein that has a similar sequence length to that of Tan but is in fact a different protein altogether. In this case, the Western blots show no new information about possible differential expression of Tan throughout pupal development of *D. americana* and *D. novamexicana*.

It would be useful to conduct further testing in the future to make sure the unexpected results from the Western blots are in fact correct. It is possible that something went wrong during the protein extraction or sample collection process. It is also possible that the specific tube with the tan put 642 antibody was made incorrectly, and that using a different tube of the antibody would give different results. It is also possible that In all, further experiments will be needed in order to see if the antibody being used here is the correct one or if a new antibody must be made so that it will bind to Tan in *D. americana* and *D. novamexicana*. In addition, if the antibody is correct and the Tan expression develops as shown in the results above, then further experiments will be needed to see if Ebony shows the same pattern of development, and to better explain the unexpected results.

Conclusion

Based on previous work by Wittkopp et al (2003), we know that *D. americana* and *D. novamexicana* express Ebony at different levels, but that Yellow expression seems constant for both species. Therefore, the hypothesis was that there would be a difference in Tan expression or *tan* mRNA levels at some point in the development of the two species. However, the results of this study have yet to show that this is the case. In the first section, the mRNA levels of *tan* were analyzed at the late pupal stage, where it has been proposed that the gene shows its activity peak, and when Yellow and Ebony seem to have the most expression. At this stage, there seems to be a high level of mRNA signal of *tan*, but the gene is expressed in very high levels equally in *D. americana* and *D. novamexicana*. The staining seems to be real and the negative control seems to work the way it should, so this suggests that the *tan* probe is binding to the correct sequence and not just nonspecific sequences throughout the cuticle. In addition, the abdominal cuticles of adults for both *D. americana* and *D. novamexicana* show pigmentation equally distributed throughout the entire abdomen. The difference in abdominal pigmentation between these species and *D. melanogaster* is consistent with the mRNA stainings, so there is nothing to suggest that the results are incorrect.

Next, the developmental rates of *D. americana*, *D. novamexicana*, and *D. melanogaster* were compared by running a developmental time series for the three species. Again, it was expected that *D. americana* and *D. novamexicana* would show different results from *D. melanogaster*, since the generation time of the two species is around two or three times as long as the generation time for *D. melanogaster*. The results showed that the developmental rate for *D. melanogaster* is in fact much faster than the

rate of development for *D. americana* and *D. novamexicana*. In addition, there seemed to be less variability in the duration time for each stage of *D. melanogaster* than in the duration time for each stage in either of *D. americana* or *D. novamexicana*. The two species, though showing higher variability, showed no difference in developmental rates from one to the other. This is also not unexpected, because the generation times for both species are approximately equal.

The one surprising result in the developmental time series was that the rates of development for *D. americana* and *D. novamexicana* were closer than expected to that of *D. melanogaster*. However, there are a number of reasons, as discussed earlier, that could explain this. Some of those reasons are that the lower temperature might have slowed down development of *D. melanogaster*, that the stages before P8 in pupal development might show a larger difference in developmental rates, and that it might take less time for *D. melanogaster* adults to lay eggs once they start the mating process. These hypotheses can all be tested in the future by conducting different staging tests and seeing what the rates of development are at younger age, or at different temperatures.

The next section of the experiment was to see if there was a difference in protein expression levels for Tan in *D. americana* and *D. novamexicana* at any stage in development. Wright et al (1991) studied the temporal and spatial expression of Yellow in *Drosophila* and found a correlation to the developmental expression of the protein and the aging process of the fly. There were two peaks of Yellow activity that they found throughout development (Wright et al 1991). Therefore it is possible that Tan also shows specific activity peaks throughout development, and that these peaks of strongest protein expression levels might occur at different times for *D. americana* and *D. novamexicana*.

However, it is hard to make any conclusive decisions about the developmental expression of Tan for *D. americana* and *D. novamexicana*, based on the results found in this study. The protein that is supposed to be Tan seems to be expressed much earlier than it should for both *D. americana* and *D. novamexicana*, but not for *D. melanogaster*, which used a separate antibody. The antibody for Tan in *D. melanogaster* seemed to work the way it should, and Tan expression was shown to be the strongest in later pupal stages. Since the Tan protein relies on the function of Ebony, it makes sense that Tan would not be expressed until Ebony is, and this occurs around stage P12 or P13 in the pupal development. Therefore it makes sense that in *D. melanogaster*, Tan expression started at low levels at stage P10, and that the strongest bands of protein expression occurred at stage P13.

However, it was surprising to see that the expression of Tan in *D. americana* and *D. novamexicana* seems to be the strongest at stages P8 and P9, while at P13 there seems to be very little expression of Tan in *D. americana*. It also does not make sense that Tan would be expressed at higher levels in *D. novamexicana* during these later stages, when Tan and Ebony should be functioning in the pigmentation pathway to produce the desired melanin. It is possible, though, that Tan is expressed higher in *D. novamexicana* because Ebony is expressed at such high levels for that species. There is a possibility that while Tan and Ebony are both expressed higher in *D. novamexicana*, the ratio of Ebony to Tan expression is higher in *D. novamexicana*, and so the lower Ebony:Tan ratio in *D. americana* leads to the darker pigmentation in that species. The Western blots for Tan protein were repeated using the same samples, and the same results were acquired, so the strange pattern of expression is not a result of loading the gel incorrectly or mislabeling

the species on the gel. It seems instead that there is either a mistake with the antibody for Tan in *D. americana* and *D. novamexicana* or that Tan is being expressed in ways that are much more different than expected.

From these results, there are a number of tests to complete for further study. The in situ hybridization seemed to work well for the late pupal stage of *D. americana* and *D. novamexicana*, unlike the antibody staining at the same stage. Therefore it seems that the *tan* probe works correctly, even though the antibody for Tan in *D. americana* and *D. novamexicana* may not. So one experiment that would be useful would be to analyze the developmental mRNA levels of expression for *tan* in the two species. Instead of collecting samples at each stage for protein extractions used for the Western blots, it would be helpful to run in situ hybridizations using samples from each stage. The in situ hybridization also only uses the abdominal cuticle, so then there wouldn't be an issue with *tan* that is being expressed but not for the pigmentation pathway. Tan is expressed in the body of the fly, in the pigmentation pathway and in the heads of flies as part of a neurotransmitter system for the eyes. So if the entire fly was used for Western blots, it was possible that some of the protein level was not due to the pigmentation protein but instead a protein with a very different function. For in situ tests, there won't be any *tan* mRNA that will be used for the neurotransmitters in the eyes, since only the abdominal cuticles are analyzed for RNA signal. Therefore, a series of in situ stainings for different stages of development would be useful in seeing the temporal expression of the *tan* gene.

The other important future task is to determine if the antibody for Tan is binding to only Tan in *D. americana* and *D. novamexicana* and if not, to find a new antibody that does so. Once we have a successful antibody, we can try the immunostaining and

Western blots again and see if the expression of Tan shows a different developmental pattern. The antibody doesn't seem to work right now for antibody stainings, since there has not been any sign of cellular stainings in all of the antibody stainings we have tried; and it is unclear if the antibody works correctly for Western blots of *D. americana* and *D. novamexicana* since the developmental series for the Western blots from this experiment are so unexpected and possibly incorrect. If it is proven that the antibody in the Western blots is binding to Tan in *D. americana* and *D. novamexicana*, and that the same temporal expression patterns are seen, then the next step would be to try a developmental series of Western blots for Ebony and see if it expresses the same pattern. In addition, further tests are needed, as described earlier, in order to try to figure out why *D. novamexicana* might be expressing higher levels of Tan than *D. americana* in the pigmentation pathway.

In all, this experiment has shown that *D. americana* and *D. novamexicana* are very closely related morphologically and genetically. There has been no new definitive evidence yet of a difference in Tan expression between the two species, although further tests are necessary, since the antibody for Tan in *D. americana* and *D. novamexicana* may not be binding correctly, and if they are then they show unexpected results about the different expressions of Tan throughout pupal development. In addition, there does not seem to be a difference in *tan* mRNA levels and the spatial arrangement of the RNA at the late pupal stages, where *tan* should be expressed the strongest. However, there is still a chance that the RNA levels could differ at a different stage, and this must be tested in the future. The developmental rate for both species is also very similar, and they both consistently age more slowly than *D. melanogaster*, as was expected. So the results of this experiment supports the idea that *D. americana* and *D. novamexicana* are very

closely related species and fails to find any conclusive evidence of different expressions of Tan protein or *tan* RNA between the two.

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