In the early 20th century, Thomas Hunt Morgan, Calvin Bridges, and colleagues began to identify mutations in *Drosophila* that produced visible phenotypes in adults. Some of these mutations have led to groundbreaking discoveries: *white* mutants verified the chromosomal theory of inheritance (Morgan 1910), *Notch* mutants (Dexter 1914; Morgan and Bridges 1916) lead to the discovery of an oncogenic signal transduction pathway and a mechanism for lateral inhibition in cell fate determination in development (Siebel and Lendahl 2017); and *Ultrabithorax* (or *bithorax*) as it was known then (Bridges and Morgan 1923)) became a founding member of the homeobox family and an important factor in anterior/posterior patterning in metazoan development (Pick 2016).

Remarkably, some of Morgan and colleagues’ mutant strains have only recently been assigned to a gene, while others still await identification. Many of these mutants fall into a few phenotypic categories like wing shape, bristle morphology or pigmentation (in eyes or body). In recent years, the *Curly* mutation (Ward 1923) was identified as an allele of the *Duox* gene, though it was identified almost 100 years prior (Hurd et al. 2015). The *straw* mutation (Morgan *et al.* 1925) was mapped to the *laccase2* gene exactly 100 years after it was discovered (Sickmann *et al.* 2017). In addition, a systematic screen has mapped many unannotated mutations, in some cases, to single genes (Kahsai and Cook 2018).

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**ABSTRACT** The pigmentation mutation *speck* is a commonly used recombination marker characterized by a darkly pigmented region at the wing hinge. Identified in 1910 by Thomas Hunt Morgan, *speck* was characterized by Sturtevant as the most “workable” mutant in the rightmost region of the second chromosome and eventually localized to 2-107.0 and 60C1-2. Though the first *speck* mutation was isolated over 110 years ago, *speck* is still not associated with any gene. Here, as part of an undergraduate-led research effort, we show that *speck* is encoded by the Arylalkylamine N-acetyltransferase 1 (*AANAT1*) gene. Both alleles from the Morgan lab contain a retrotransposon in exon 1 of the *RB* transcript of the *AANAT1* gene. We have also identified a new insertion allele and generated multiple deletion alleles in *AANAT1* that all give a strong *speck* phenotype. In addition, expression of *AANAT1* RNAi constructs either ubiquitously or in the dorsal portion of the developing wing generates a similar *speck* phenotype. We find that *speck* alleles have additional phenotypes, including ectopic pigmentation in the posterior pupal case, leg joints, cuticular sutures and overall body color. We propose that the acetylated dopamine generated by *AANAT1* decreases the dopamine pool available for melanin production. When *AANAT1* function is decreased, the excess dopamine enters the melanin pathway to generate the *speck* phenotype.
In Drosophila, pigmentation of the adult cuticle is a combination of melanin and sclerotin, which are end-products of branches of a shared biosynthetic pathway (Figure 1, reviewed in (Yamamoto and Seto 2014) and (Massey and Wittkopp 2016)). The pigmentation pathway begins with the sequential modification of L-tyrosine to L-Dopa by Tyrosine Hydroxylase (encoded by the pale gene) and to Dopamine by Dopacarboxylase (encoded by the Ddc gene). At dopamine, the pathway branches onto three routes: the melanin pathway, the NBAD sclerotin pathway and the NADA sclerotin pathway. Both L-Dopa and dopamine function as precursors for the extracellular production of melanin through quinone intermediates of the melanization pathway causing a dark body color. Finally, dopamine may be converted to N-acetyldopamine (NADA) by the activity of Arylalkylamine N-acetyltransferase 1 (AANAT1) (previously called Dopamine Acetyltransferase, Dat). NADA serves as an intermediate in the formation of melanin and sclerotin, which are end-products of branches of a shared biosynthetic pathway (Figure 1) (Yamamoto and Seto 2014) and (Massey and Wittkopp 2016)). The pigmentation pathway branches onto three routes: the melanin pathway, the NBAD sclerotin pathway and the NADA sclerotin pathway. Both L-Dopa and dopamine function as precursors for the extracellular production of melanin through quinone intermediates produced by the multicomponent oxidase activity of the straw (laccase) gene (Riedel et al. 2011). The yellow gene is required to produce melanin in the extracellular space but its exact activity in the process is not known (Hinaux et al. 2018) though paralogs yellow-f and yellow-f2 are dopamine isomerases (Han et al. 2002); one of the last steps in melanin synthesis. In the NBAD sclerotin pathway, the enzyme NBAD synthase (encoded by the ebony gene) combines the dopamine with beta-alanine and the product, N-Beta-alanyldopamine (NBAD) is secreted into the extracellular space where it is also converted to a quinone by straw. The beta-alanine is the product of the Aspartate decarboxylase enzyme (encoded by the black gene). Loss of ebony or black allows more dopamine to enter the melanization pathway causing a dark body color. Finally, dopamine may be converted to N-acetyldopamine (NADA) by the activity of Arylalkylamine N-acetyltransferase 1 (AANAT1) (previously called Dopamine Acetyltransferase, Dat). NADA secreted into the extracellular space may also be converted to a quinone by straw and used in the formation of NBAD sclerotin, which is colorless.

The Drosophila melanogaster AANAT1 enzyme is a member of the GCN5-related N-acetyltransferase (GNAT) superfamily and transfers an acetyl group from acetyl-CoA to biogenic amines including dopamine, serotonin, and others via a sequential binding mechanism (Cheng et al. 2012; Dempsey et al. 2014a). The AANAT1 locus has two splice isoforms (RA and RB) and is expressed throughout development in a subset of gut, neuronal, and glial cells (Brodbeck et al. 1998; Hintermann et al. 1995; Hintermann et al. 1996; Croset et al. 2018; Davla et al. 2019). A partial loss of function allele (AANAT1<sup>ra</sup>), which shows decreased levels of isoform RA, shows no pigmentation phenotype but has behavioral defects in sleep (Ganguly-Fitzgerald et al. 2006; Shaw et al. 2000; Davla et al. 2019). Strong loss of function phenotypes in Tribolium (flour beetle) (Noh et al. 2016), Bombyx (silk moth) (Dai et al. 2010; Zhan et al. 2010), Zootermopsis (termite) (Masuoka and Maekawa 2016), and Oncopeltus (milkwedge bug) (Liu et al. 2016) show increased melanization, and AANAT1 has also been identified as the locus responsible for interspecies pigment differences in Drosophila (Ahmed-Braimah and Sweiertag 2015).

In a project initiated in an undergraduate laboratory course, we have mapped the speck mutation using genetic complementation and identified lesions in the gene encoding Arylalkylamine N-acetyltransferase 1 (AANAT1). Both speck<sup>1</sup> and speck<sup>2</sup> contain the same 412 retrotransposon, though the speck<sup>2</sup> phenotype is stronger than speck<sup>1</sup>. We have generated new deletion alleles that show a darkening of the adult cuticle and additional phenotypes. All of these phenotypes can be phenocopied by RNAi and rescued by expression of a transgene. Loss of the AANAT1 activity would allow more dopamine to enter the melanin biosynthesis and/or NBAD sclerotin biosynthesis pathway and produce darker animals in a mechanism similar to other insects.

### MATERIALS AND METHODS

#### D. melanogaster culture, handling, and crosses

All stocks were obtained from the Bloomington Drosophila Stock Center at the University of Indiana, except for the UAS-Bm-aaNAT/TM3 (obtained from Teruyuki Niimi, Nagoya University) and the y<sup>+</sup> w<sup>1118</sup> and y<sup>+</sup> w<sup>1</sup>; bw<sup>1</sup> speck<sup>1</sup> zip<sup>2</sup>/SM6a (obtained from Dan Kiehart, Duke University). Crosses were performed and maintained at 25°C on standard cornmeal/molasses/agar based fly medium.

#### Minos reversion crosses

Flies of w<sup>1118</sup>, Mi[ET1]AANAT1<sup>MB02738</sup> were crossed to w<sup>1118</sup>; no<sup>910v</sup>/SM6a, P[hsILMIt]2.4, and the resulting larvae were heat shocked daily for 1 hr in a 37°C water bath over 3-4 days. Single males or two virgin females of w<sup>1118</sup>; AANAT1<sup>MB02738</sup>/SM6a, P[hsILMIt]2.4 were crossed to w<sup>1118</sup>, Df(2R)BSC356/SM6a. Progeny males in the next generation that lacked the mini-w<sup>+</sup> eye color associated with P[hsILMIt] were scored for the presence of GFP and any speck phenotype. Ten independent lines (6 speck<sup>+</sup>, 4 speck<sup>-</sup>) were balanced over CyO.

#### Adult tissue mounts and photography

Anesthetized 2-5 or 7 day-old male flies were photographed with a Nikon D300S camera mounted to a Leica M2Z12 stereomicroscope, using a ring light for illumination. For time-lapse movies, newly eclosed adults were affixed at the notum to a micro-injection needle, which was mounted on an ~1-cm cube of modeling clay. An image was taken every 5 sec using Camera Control Pro 2 software (Nikon) for a total of 2500 images. Individual jpg images were combined into an .mpg movie at 30 frames per second using Time Lapse Assembler software Version 1.5.3 (by Dan Bridges 2012).

#### Molecular biology

Genomic DNA was purified from wild type and mutant flies, using the Qiagen DNeasy Blood and Tissue Kit (#69504) and stored at -20°C after isolation. PCR amplification of long products was completed using Qiagen LongRange PCR Kit (#206401). Sequencing was performed by Eton Biosciences (Research Triangle Park, NC).

#### Whole-genome sequencing

Two libraries consisting of genomic DNA purified from males of the genotypes IP3K2<sup>2<sup>-<sup>1</sup></sup></sup>; SM6a, speck<sup>2</sup>+ and from t<sup>1</sup> v<sup>1</sup> m<sup>74</sup> IP3K2<sup>2<sup>-<sup>74</sup></sup></sup>; speck<sup>2</sup> bs<sup>2</sup>+ were used as template for HiSeq 4000, 150bp, paired-end sequencing (BGI Tech). Over 36.6 million reads for each library produced ~5.5 Gigabases of sequence for each. A 20 kb region spanning the AANAT1 region was assembled and analyzed using CONSED, version 29.0 (Gordon and Green 2013) and minimap2, version 2.17-r941 (Li 2018) and visualized using Tablet, version 1.19 (Milne et al. 2012).

#### CRISPR mutations

Guide RNAs corresponding to two regions of the AANAT1 gene were designed according to the method described in (Gratz et al. 2013). Phosphorylated oligonucleotides were obtained (IDT DNA), annealed and ligated into a linearized, dephosphorylated pU6-BbsI-chiRNA plasmid. A mix of both plasmids corresponding to the left and right breakpoints were injected into M[vas-Cas9]ZH-2A, w<sup>1118</sup> /FM7c by Model System Injections (Durham, NC). Injected G0 adults were crossed singly to w<sup>1118</sup>, Mi[ET1]AANAT1<sup>MB02738</sup>, and the resulting progeny were scored for a speck phenotype. Three adults showing a speck phenotype were obtained from 66 crosses,
and new speck alleles were balanced over CyO, homozygosed, and molecularly characterized by PCR amplification and sequencing.

Data availability
Genbank accession numbers and annotated sequences of the Minos excisions for speckT1, speckG1, and speckD1 and CRISPR deletions speck31B, speck35A, and speck47B are shown in Supplemental Figure 1. Whole genome sequencing reads for sp2 bs2 and SM6a have been deposited in SRA with the accession number: PRJNA643549. A table of primer sequences used is included as a Supplemental Table. Drosophila strains/reagents are available upon request. Supplemental material available at figshare: https://doi.org/10.25387/g3.12357578.

RESULTS
Though undergraduate scientists have been a staple of Drosophila research groups since Alfred Sturtevant worked for Morgan, only relatively recently has this research been extended to the classroom. Multi-year projects in lab courses at the University of California, Los Angeles identified mutations responsible for eye development (Call et al. 2007) and cell lineage analysis (Olson et al. 2019) and a multi-year, multi-University consortium directed from Washington University in St. Louis had over 900 undergraduate students finish and annotate the dot chromosomes of a number of Drosophila species (Leung et al. 2015). Other Course-based Undergraduate Research Experiences (CUREs) have gained more prominence at Universities.
as faculty identify the advantages of such an approach (Shaffer et al. 2014) (see CURENet: curenet.cns.utexas.edu for additional information), and using genetic complementation to characterize mutants derived from a screen has been quite successful (Bieser et al. 2019; Stamm et al. 2019). The success at the University level has led to this approach moving into high schools, where one collaboration between a high school and University yielded a LexA Drosophila enhancer trap collection (Kockel et al. 2016).

The creation of a molecularly defined deficiency kit (Cook et al. 2012; Roote and Russell 2012) and two separate X chromosome duplications sets (Cook et al. 2010; Venken et al. 2010) allowed the simple, rapid mapping of Drosophila melanogaster mutations to a precise genomic interval by complementation. In combination with the ongoing transposable element mutagenesis from the Gene Disruption Project (Bellen et al. 2011), we hypothesized that a few undergraduate students could map mutations with adult visible phenotypes to a single gene within a semester. To decide which mutations to map first, we took a historical perspective and chose the four oldest mutations that FlyBase designated as unannotated (not associated to a transcription unit), that still maintained the adult, visible phenotype as described, and had stocks available: speck (speck, first identified in 1910), curved (cu, 1911), spread, (sprd, 1913), and till (tt, 1915). We found that cu1 contained a ~7 kB Element retrotransposon (position 15,953,913, Genome annotation release R6.27) in an exon of the Stretchin-Mlick (Stn-Mlick) gene as described in Rodriguez, 2004 and failed to complement an insertion in Stn-Mlick (pBac[PB]Strn-Mlick{c2826}). We found that the spread stock died prior to 1923 (Bridges and Morgan 1923) and that all stocks listed as sprd1 are likely mis-annotated. We mapped tt1 to a defined molecular region and speck to a single gene, which we further characterized outside of class.

History of the speck mutation

Thomas Hunt Morgan isolated the first speck mutation in March 1910. However, that first speck mutant “was set aside in order that more time might be given to the study of the sex-linked eye-color white which had appeared in April 1910” (Bridges and Morgan 1919). A second, phenotypically stronger allele of speck was identified by Morgan in May 1911 (occasionally referred to as olive speck in their writing due to its slightly darker body color), and unfortunately, the 1910 stock was discarded. This 1911 olive speck mutation is what is currently known as speck1. A third speck allele was identified in June 1925 by Calvin Bridges from a stock (al b pr cn vg a speck1) that had an unusually dark body color than would be expected of a black speck double mutant. Subsequent crosses and tests led Bridges to conclude that the chromosome carried a new, stronger speck allele, which he named speck2 (Bridges, Unpublished Results). The speck phenotype was described as a dark, melanized region at the hinge (axil) of the wing and a slight darkening of the body color. Bridges also noted “darker, brooch-shaped” region at the former anal region of the pupal case. As the adult phenotype was easy to score and speck mapped to the distal region of the right arm of chromosome 2 (2-107.0), speck has been a useful tool for genetic recombination mapping since the early 20th century. Because speck2 adults have a slightly darker body color, speck was predicted to function in the melanization or sclerotinization pathway (Wright 1987).

Mapping the speck mutation and generating new alleles

The easiest speck phenotype to score is the melanized region at the axil of the wing (Bridges and Morgan 1919) and we chose this as the phenotype to score in complementation tests. At the start of the course, there were two available alleles of speck: speck1 and speck2, and speck2 is also found on the SM5 and SM6 balancer chromosomes. Previous mapping had placed speck in the cytological region of 60C1,2; which roughly corresponds to more than 60 kb of DNA containing approximately 12 protein-coding transcripts (Peng and Mount 1990). To account for the variance in breakpoints between cytologically defined deficiencies and molecularly defined deficiencies, we tested the complementation of both speck1 and speck2 with six deficiency strains that covered cytological region 60B8 to 60D14 (Figure 2A). We found that two of the six deficiency strains failed to complement speck (Df(2R)BSC356 and Df(2R)BSC155), molecularly defining the speck interval as between the left breakpoint of Df(2R) BSC155 (by inclusion) and the left breakpoint of Df(2R)BSC780 (by exclusion), a region containing approximately 26 protein-coding transcripts (Figure 2B).

Genetic interactions with speck helped to further narrow the list of candidate genes. The suppressor of sable, su(sable), mutation suppresses aspects of the speck1 phenotype (Warner et al. 1975), and su(sable) also suppresses phenotypes caused by 412 or roo retrotransposon insertions in a number of genes. In situ hybridization to polytene chromosomes showed that a 412 element was present in the genomic region near speck (Searles and Voelker 1986), indicating that the speck1 allele may be derived from a 412 insertion.

In a lucky coincidence, the speck1 allele was sequenced by whole genome sequencing—it was present in the isogenic strain (y; c111 bv1 speck) used for the Drosophila Genome Project (Adams et al. 2000) and is therefore displayed as the reference strain in the genome browsers in Flybase. Two 412 elements are present in the region defined by the deficiency breakpoints: 412[1]881 and 412[2]882 (Figure 2B). The 412[1]881 element is not present in any speck1 strains (by PCR or analysis of whole-genome sequencing). It is present in multiple stocks of both speck1 and speck2. The 7,566 bp 412[2]881 element (Kaminker et al. 2002) is inserted in the 5’ UTR in exon 1 of the gene encoding Arylalkylamine N-acetyltransferase 1 (AANAT1), splice isoform RB. An allele of AANAT1, AANAT1lo (Figure 2C) has been shown to cause a decrease in the AANAT1-RA product; however, we find that AANAT1lo fully complements all speck alleles. Figure 2C shows the AANAT locus as it appears in wild type genomes, which matches the gene structure of the locus described previously (Brobeck et al. 1998).

We attempted to identify the molecular difference between speck1 and speck2. Whole genome sequencing of two different strains with speck2 alleles were sequenced as heterozygotes with the prediction that changes in the AANAT1 locus that were shared between them would warrant further examination as being the speck2 lesion. Assembly of the AANAT1 locus from both genotypes using Consed (Gordon and Green 2013) and comparison to the speck1 reference strain did not yield any candidate changes (data not shown). In addition, examination of SNPs between balancers carrying speck2 and the reference strain carrying speck1 showed no SNPs within the AANAT1 locus (Miller et al. 2018). Because there were no changes in the genomic interval that short-read, whole genome sequencing could identify, we looked to see if 412[2]881 had a second retrotransposon insertion, similar to AANAT1lo (Brobeck et al. 1998). However, a PCR product spanning the 412{881} insertion was not identifiable, we looked to see if 412{881} had a second retrotransposon insertion, similar to AANAT1lo (Brodbeck et al. 1998).
We obtained more definitive evidence that speck mutants disrupt AANAT1 from the study of a strain containing a Minos insertion, AANAT1MB02738. This insertion is in the same exon as the 412{}881 insertion in speck1 and speck2 and displays a strong speck phenotype (Figures 2C and 3D) and fails to complement speck1 and speck2 alleles. To test whether the Minos insertion causes the speck phenotype, we mobilized the Minos insert with a hs-Minos transposase (SM6a, P[hs-MiTpase]) (Metaxakis et al. 2005) and recovered 10 unique lines that lacked the transgenic marker associated with the Minos ET1 vector (GFP). Of these 10 lines, 4 maintained the speck phenotype as homozygotes (speck-), but 6 fully reverted the speck phenotype to wild type (speck+). One allele, speckT1, reverted the speck phenotype and has a wild-type sequence at the insertion site (Figure 3E). One of the speck- alleles, speckG1, was an imprecise excision that deleted over 500 bases, including the AANAT1-RB transcription start site (Figures 2C and 3F). In addition, we identified an unusual allele from this mobilization, as well. The speckT1 allele (not shown) reverts the speck phenotype to wild type and has removed a large wild-type sequence at the insertion site (Figure 3E). One of the RNAi regions (JF02142 and HMS01617) are located at the 3’ end of the transcript and can also produce a speck phenotype. The vertical green lines through the transcripts show the approximate locations of the gRNAs used to make the CRISPR alleles.

To make null alleles that would remove both AANAT1 splice isoforms, we attempted to make deletion alleles using CRISPR (Gratz et al. 2013). We designed two Cas9 guide RNA binding sites: one in intron 1 of the AANAT1-RB isoform and another in the 3’ UTR shared by both isoforms. Two Cas9 cuts and non-homologous end-joining would create a deletion lacking all the functional domains of AANAT1. After injections of the two guide-RNA plasmids into Cas9 expressing embryos, we crossed individual surviving adults to the w; AANAT1MB02738 stock and scored their progeny for a speck phenotype. Out of 66 crosses, we identified 3 CRISPR derived speck alleles and sequenced PCR products that spanned the lesions in each. The strongest allele, speck35A, removes exons 1 and 2 of AANAT1-RB and exon 2 of AANAT1-RA (Figure 2C). There should be no protein generated from the AANAT1-RB isoform (since its transcription should never be initiated), and since exon 2 of AANAT1-RA has the translation initiation codon, creation of a protein product from AANAT1-RA in speck35A would necessitate an exon skipping event from exon 1 to 3, and for the first AUG in exon 3 to be in the correct
frame (it is not). Like AANAT1\(^{G1}\), the CRISPR-derived allele speck\(^{47B}\) also removes only exon 1 of AANAT1-RB and shows a speck phenotype (Figure 2C and 3G). The speck\(^{31B}\) CRISPR allele removes exon 2 from both AANAT1 isoforms (Figure 2C) and shows a speck phenotype (data not shown).

Additionally, we found that we could replicate the speck phenotype using RNAi against the AANAT1 gene. Two different AANAT1 hairpin constructs under UAS control (Figure 2C) produced a speck phenotype. While we also see speck\(^2\) alleles as slightly darker than speck\(^1\), we found that AANAT1\(^{MB02738}\) and some deletion alleles are much darker and present additional phenotypes. We find that the AANAT1\(^{3b}\) allele (Figure 4B) has no obvious ectopic pigmentation and looks quite similar to a wild type strain, w\(^{1118}\) (Figure 4A). The AANAT1\(^{MB02738}\) allele (Figure 4C) shows not only strong ectopic pigmentation in the wing hinge, but additional areas. The intersection of the scutellum and mesothoracic laterotergite shows a highly melanized spot (arrowhead in enlarged image) and the joints also show ectopic pigmentation (arrow). In addition, the sutures between cuticle plates on the lateral body wall are more pronounced, however this may be caused by the overall darkened cuticle. This darkened cuticle is more severe than found in the natural variation in D. melanogaster (Werner et al. 2018). The CRISPR derived alleles speck\(^{31B}\) and speck\(^{35A}\) show a very similar phenotype to AANAT1\(^{MB02738}\) (Figures 4D and 4E) with regard to body color, wing hinge, scutellum, and leg joint pigmentation. The speck\(^{47B}\) allele, however, shows a phenotype more similar to speck\(^1\), showing only the wing hinge phenotype.

The wing hinge and body color are not the only described phenotypes of speck mutants. Both Bridges (Bridges, Unpublished Results) and Waddington (Waddington 1942) described a dark anal pad of the pupal case during pupariation. Because this phenotype had been described, but never shown in a publication, we documented the anal pad phenotype of

**Figure 3** Mutations in the AANAT1 gene cause the speck phenotype of ectopic pigmentation in the wing hinge and darker body color. Adult males are shown with anterior to the left and dorsal up. A) A w\(^{1118}\) male shows the wild type pigmentation at the ventral wing hinge region. B.) The speck\(^1\) allele (cn\(^1\) bw\(^1\) speck\(^1\)) shows a “speck” of pigmentation at the ventral wing hinge. C.) The speck\(^{31b}\) allele (Df(2R) BSC356/SM6a) shows the dark speck at the wing hinge and a slightly darker body color. D.) An insertion allele of AANAT1 (Mi{ET1}AANAT1\(^{MB02738}\)) shows a darker body color and a pronounced wing hinge spot. E.) Mobilization of the insertion in D can revert the speck phenotype (speck\(^{7f}\) F.) An imprecise excision of the insertion in D that removes an AANAT1 transcriptional start site produces a speck phenotype (speck\(^{47B}\)). G.) A CRISPR allele (speck\(^{47B}\)) that deletes 1218 bp shows an intermediate speck phenotype. H. and I) Knock-down of the AANAT1 gene by two different GAL4 drivers and two different hairpin locations produce a speck phenotype (H: Tubulin-GAL4, UAS-dicer2; UAS-AANAT1\(^{MB02738}\) and I: apterous-GAL4; UAS-AANAT1\(^{F02142}\)).
speck mutants. We find that compared to wild type (Figure 5A) the anal pad in both speck1 (Figure 5B) and speck2 (Figure 5C) are substantially darker. This ectopic pigment is also present in AANAT1MB02738 (Figure 5D) and can be reverted to wild type (Figure 5E) after mobilization of the Minos insertion. Furthermore, ubiquitous expression of a small hairpin against the AANAT1 gene can phenocopy speck in the anal pad of pupae (Figure 5F).

**Rescue and ectopic expression**

We wanted to determine if expression of a related AANAT gene could rescue the pigmentation phenotype of speck mutants. Expression of the Bombyx mori ortholog of the Drosophila AANAT1 gene has been shown to decrease pigment production in Drosophila when misexpressed via heat shock (Osanai-Futahashi et al. 2012). We used this transgenic construct (UAS-Bm aANAT) to rescue the speck phenotype. Flies heterozygous for the AANAT1MB02738 allele (w1118; AANAT1MB02738) show not only ectopic pigmentation at the wing hinge region, but at the region between the scutellum and the mesothoracic preepisternum (arrowhead), and also the leg joints (arrow). In addition, they show an overall darker pigment to the thorax, which makes the regions between portions of the body wall more evident. D and E) Males of w1118; speck31B (D) and w1118; speck47B (E) show a similar phenotype to those in C. F.) Males of w1118; speck47B show only the wing hinge phenotype.

**DISCUSSION**

We have mapped the speck mutation, first identified by Thomas Hunt Morgan in 1910, to the AANAT1 gene and found that both Morgan’s
(speck1) and Bridges’ (speck2) alleles are retrotransposon insertions in the 5’ UTR of one of AANAT1’s two transcripts. However, no obvious differences in sequence between their two alleles were identified. A transposon mutagenesis screen almost 100 years after speck was discovered produced a Minos insertion in the same region that fails to complement speck and gives a similar phenotype. Excision of that Minos element can revert the speck phenotype, indicating that the insertion causes the phenotype. Deletion alleles that remove parts of both transcription units also give a speck phenotype, indicating that AANAT1 null flies are adult viable, fertile and have a speck pigmentation phenotype. Because we find mutagenic insertions in AANAT1 only in speck mutations, that removal of the insertions can revert the phenotype to wild type, that deletions of AANAT1 produce a speck phenotype and that the phenotype can be rescued by a transgene, we can conclude that speck is a loss of AANAT1; that AANAT1 is the speck gene.

How does speck fit into the Drosophila cuticular pigmentation pathway?
While AANAT1 has been known in the cuticular pigmentation pathway for some time, the speck phenotype is not what would have been predicted from a mutation that causes increases in melanin and NBAD sclerotin. As seen in Figure 1, ebony mutants would have a loss of tan pigment due to the loss of NBAD sclerotin, but increases of black/brown melanin and colorless NADA sclerotin due to dopamine being shunted into those pathways. One would predict that speck

Figure 5 The pupal case of speck mutants display excess pigmentation at the anal pad. All images are of the posterior ventral sides of pupal cases following eclosion. A.) An Oregon R pupal case shows normal posterior pigmentation. B.) A speck1 mutant (cn1 bw1 speck1) shows the anal pad region is darkly pigmented. C.) A speck2 mutant (speck2 bs2) shows a similar dark band as speck1 and the pupal case overall is slightly darker. D.) An insertion in AANAT1 (AANAT1MB02738) shows a similar phenotype and can be reverted to wild type upon mobilization (E). F.) Knockdown of AANAT1 via RNAi can reproduce the phenotype (Tubulin-GAL4, UAS-Dicer2; UAS-AANAT1MB02738).

Figure 6 The Bombyx mori aaNAT gene can rescue speck mutations, but causes decreased pigmentation. Adult males are shown with anterior to the left and dorsal up. A.) A male heterozygous for the AANAT1MB02738 allele shows wild type pigmentation (w1118; AANAT1MB02738/+) B.) The speck phenotype is observed in the AANAT1MB02738/speck2 genotype (w1118; AANAT1MB02738/SM6a) C.) Ubiquitous misexpression of the Bombyx mori aaNAT gene causes a loss of pigmentation (w1118; AANAT1MB02738/+; TubulinGAL4, UAS-Bm-aaNAT/+). D.) Ubiquitous misexpression of the Bombyx mori aaNAT rescues the speck phenotype (w1118; AANAT1MB02738/SM6a; TubulinGAL4, UAS-Bm-aaNAT/+). E.) The speck phenotype in the wing hinge is still visible in genotypes that reduce pigmentation, such as yellow. (y'/w; bw1; speck1, zip2/SM6a).
mutants would lose no visible pigments from loss of NADA sclerotin, but would have increases of black/brown melanin and tan NBAD sclerotin pigment. So how could speck mutants be lighter in body color than ebony mutants?

Since ebony (and black) mutants are much darker than speck mutants, there may be differences in the amount of NBAD vs. NADA sclerotin in different parts of the cuticle. Presuming both ebony and speck mutants have the same amount of L-dopa and dopamine going into the choice points as reviewed in Figure 1, the strengths of the pathways could be different. If the dorsal thorax utilized the NBAD sclerotin pathway much more than the NADA sclerotin pathway, loss of ebony would send a substantial amount of precursors into the NADA pathway (which is colorless) and black/brown melanin pathways giving the ebony mutants their namesake color. So ebony mutants are very dark because they have moved enough dopamine out of the NBAD sclerotin pathway and into the melanin pathway to make very dark flies. However, if the amount of NADA sclerotin in the dorsal thorax was low, loss of speck would provide less dopamine for the NBAD and melanin pathways. So speck mutants are a little darker but not as dark as ebony. In other regions of the adult cuticle, like the wing hinge and leg joints, this might be reversed, implying those regions have higher concentrations of NADA sclerotin than NBAD sclerotin and have more precursors to send to the pathways yielding black/brown and tan pigments from increased melanin and NBAD sclerotin.

What might these differences reveal? The most obvious difference is that the dorsal cuticle is hard and rigid and the wing hinge and leg joints are soft and pliable. Might the ratio of NBAD vs. NADA sclerotin make a difference in cuticle hardness? Drosophila black mutants show a significant increase in puncture resistance when injected with beta-alanine to rescue the black phenotype (Jacobs 1985). A Blattella (cockroach) strain that had decreased amounts of beta-alanine also showed decreased puncture resistance (Czapla et al. 1990) and a similar result was found with Tribolium (Roseland et al. 1987). Though this is an interesting correlation, many factors play a part in cuticular hardness such as thickness, protein and chitin composition, and others (reviewed in Anderssen 2012).

Another possibility, though is redundancy. Similar to yellow that has 12 yellow-related genes in the Drosophila genome (Drapeau 2001), there are seven AANAT-like genes in the Drosophila genome in addition to AANAT1 (Amherd et al. 2000). It is possible that one or more of these genes could also function in sclerotization, and that AANAT1 might not act alone. One of these AANAT-like genes, AANATL2, has been shown to be present in adults and catalyze the formation of long-chain acylserotonins and acyldopamines (Dempsey et al. 2014b); however, this seems unlikely to function in the NADA sclerotin pathway.

How does the speck Drosophila phenotype differ from other insects?

We find that strong speck alleles in Drosophila have ectopic melanin formation in regional positions and a slightly darker cuticle in adults. These locations in adults coincide with a slightly darker cuticle in the exoskeleton where flexibility is required, such as leg joints, wing hinges and suture locations between cuticle plates. Only the strongest of these phenotypes can be replicated by RNAi. In silkworms (Bombyx mori), mutations show an overall body darkening and regional melanization in the larval head, thoracic legs, and anal plate (Dai et al. 2010; Zhan et al. 2010). In adult milkweed bugs (Oncopeltus), no change in adult cuticle pigmentation was detectable by RNAi, though ectopic melanization was found in regions of the proximal hindwing (Liu et al. 2016). Expression analysis showed that this region was the only position where AANAT was expressed in either the forewing or hindwing. Reduction of AANAT by RNAi in American cockroaches (Periplaneta) showed no forewing or hindwing phenotypes, but it did show increased melanization in an unpigmented region of the T1 plate (Liu et al. 2016). In termites (Zootermopsis), the reduction of AANAT by RNAi caused intense melanization of the anterior head and mandibles (Masuoka and Maekawa 2016). Red flour beetles (Tribolium) injected with RNAi against AANAT showed a combination of phenotypes: increased melanization in adult body wall cuticle, elytra, and hindwings (Noh et al. 2016).

Common phenotypes seen across these insect species fall into two categories: broad overall darkening of the adult cuticle and regions of intense melanin localization. While some phenotypic differences can be explained by the variability of RNAi phenotypes and species-specific development, some commonalities are striking. The loss-of-function mutations in Drosophila and Bombbyx both caused overall body darkening and more intense pigmentation in legs and anal plates. Ectopic pigmentation was found in the proximal, posterior region of the hindwing in both Oncopeltus and Tribolium. It will be interesting to see how the expression pattern of AANAT1 during cuticular tanning in Drosophila relates to its pigmentation phenotype.

Possible defects in neuronal signaling in speck mutants?

In Drosophila, nervous system expression of AANAT1 is typically associated with the inactivation of monoamine signaling by acetylation. In vertebrates, dopamine signaling is inactivated by a class of enzymes called monoamine oxidases (MAOs) that have, so far, not been identified in insects (reviewed in Yamamoto and Seto 2014). Localization of transcripts and protein has placed speck in astrocytes in adult Drosophila brains, where it could function to inactivate monoamine signaling in a similar mechanism to ebony (Croset et al. 2018; Suh and Jackson 2007; Davla et al. 2019). With the publication of the adult Drosophila brain “connectome” (Zheng et al. 2018; Xu et al. 2020), the identification of which synapses inactivate biogenic amines via AANAT1 acetylation may soon be deduced. Weak alleles of speck show a defect in sleep (Shaw et al. 2000; Ganguly-Fitzgerald et al. 2006; Davla et al. 2019), as well as changes in monoamine levels (Davla et al. 2019), but it is not known how its expression pattern relates to the emerging network of sleep in Drosophila (Bringmann 2018). speck might affect sleep by more than inactivating biogenic amines. In vertebrates, AANAT1 orthologs have been termed a “Timezyme” (Klein 2007) because its rhythmic night-time expression and/or activity are the rate-limiting steps in the synthesis of melatonin at night (reviewed in Saha et al. 2018; Zhao et al. 2019).

As in vertebrates, melatonin is present in Drosophila (Finocchiaro et al. 1988) with levels rising at night (Callebert et al. 1991; Hintermann et al. 1996), and AANAT1 is thought to be the serotonin acetyltransferase used in its synthesis. Neither speck mRNA or protein undergoes daily expression cycles in whole-head extracts (Brodebeck et al. 1998); however, melatonin synthesis might only occur in a small subset of cells, and cycling might be undetectable in whole head extracts. Both speck mRNA and protein have been identified in a small subset of serotonergic neurons in adult brains (Croset et al. 2018; Davla et al. 2019), but it is not known if these neurons synthesize melatonin. Interestingly, melatonin synthesis is lost in
mutants of the circadian rhythm gene period (Callebert et al. 1991). In the Chinese Tarso Moth (Antheraea pernyi), the expression of an ortholog to AANAT1 is under the control of clock genes in a subset of neurons, and RNAi against that AANAT lead to changes in photoperiodism and decreased melatonin (Mohamed et al. 2014).

This project was the outcome of a research-based undergraduate course. Three students with no knowledge of Drosophila genetics or development mapped the speck mutation to the AANAT1 gene within one semester, and three additional students followed up on that discovery. The students acquired valuable skills in a multitude of areas including: science history, literature searches, genetics, molecular biology, photomicroscopy, writing, and scientific reasoning. While this project mapping historical mutants was successful, any number of similar projects could be designed where students work to solve problems and make discoveries.

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