Pigmentation loci as markers for genome editing in the Chagas disease vector

*Rhodnius prolixus*

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**Running title:** Kissing bug colors
Abstract

The kissing bug *Rhodnius prolixus* is a major vector for Chagas disease in the Americas, and also considered as the primary model for functional studies. Prospective transgenic approaches and genome editing strategies hold great promise for controlling insect populations as well as disease propagation. In this context, identifying visible genetic markers for transgenic methodologies is of paramount importance to advance the field. Here we have identified and analyzed the function of putative cuticle and eye color genes by investigating the effect of gene knockdown on fertility, viability, and the generation of visible phenotypes. Synthesis of the dark, yellow and tan pigments present in the cuticle of most insects depends on the function of key genes encoding enzymes in the tyrosine pathway. Knockdown of the *R. prolixus yellow* and *aaNAT/pro* orthologs produces striking alterations in cuticle color. Surprisingly, knockdown of *ebony* does not generate visible phenotypes. Since loss of *ebony* function results in a dark cuticle in several insect orders, we conclude that *R. prolixus* evolved alternative strategies for cuticle coloration, possibly including the loss of a pigmentation function for an entire branch of the tyrosine pathway. Knockdown of the *scarlet* and *brown* genes - encoding ABC transporters - alters cuticle and eye pigmentation, implying that the transport of pigment into proper organelles is an important process both for cuticle and eye coloration in this species. Therefore, this analysis identifies for the first time potential visible markers for transgenesis in a hemipteran vector for a debilitating human disease.
Author Summary

The hemipteran *Rhodnius prolixus* - also known as a kissing bug - is a main vector transmitting the parasite *Trypanosoma cruzi*, the causative agent of Chagas disease, a debilitating infection estimated to affect more than 6 million people in Central and South America. In order to limit disease spread, an important measure is insect vector control. However, kissing bugs - like other insects - develop resistance to insecticides. Alternative strategies based on transgenesis and the recently developed CRISPR-based genome edition hold great promise to control vector population or generate parasite-resistant insects. For these approaches to be feasible in *R. prolixus*, it is critical to identify visible phenotypic markers. Here we identify and describe several genes controlling cuticle and eye pigmentation that are well-suited putative landing sites for transformation strategies. Among these, loss-of-function mutations in the ABC transporter encoding *scarlet* and the tyrosine pathway enzyme encoding *aaNAT/pro* generate striking and easily visible phenotypes. Importantly, the knockdown of these genes does not affect insect viability and fertility under laboratory conditions. Our results suggest that *R. prolixus* has developed alternative strategies for cuticle coloration involving the loss of an entire branch of tanning loci, while the other branch producing cuticle patterns by generating non-pigmented areas has gained critical importance.

**Keywords:** eye pigment, melanin, cuticle, *Rhodnius prolixus*, Chagas disease, hemiptera
Introduction

Pigmentation is largely recognized as an evolutionarily selected trait in insects and other metazoans, with important functions in mate choice, camouflage, thermoregulation and resistance to desiccation and infection, among others [1]. Mutations in genes implicated in pigment synthesis pathways were first identified in *Drosophila melanogaster*, such as the eye color mutant *white* (*w*, [2,3]) and cuticle color mutants like *yellow* (*y*, [4,5]). Orthologs encoding pigment pathway enzymes have also been identified in several insect orders aside from Dipterans. Among Hemiptera (true bugs), cuticle coloration has been functionally analyzed in three plant eating species: *Oncopeltus fasciatus* [6,7], *Acyrthosiphon pisum* [8] and *Nilaparvata lugens* [9]. However, the Hemiptera order also harbors a great number of blood feeding insects. Kissing bugs, including species belonging to the Triatoma, Panstrongylus and Rhodnius genera, display a range of cuticle and eye color patterns, yet the evolutionary conservation of the pigment pathway in these species has not been investigated.

*Yellow* (*y*) is part of a highly conserved modular gene network that controls melanin and sclerotin production for cuticle coloration and sclerotization (Fig 1A). This biosynthetic pathway starts with hydroxylation of phenylalanine to tyrosine, followed by hydroxylation to dihydroxyphenylalanine (DOPA) [10], by the enzymes phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH), respectively. DOPA is the substrate for the DOPA black melanin branch, and is also converted to Dopamine by DOPA decarboxilase (DDC), the substrate for the Dopamine black/brown melanin branch. *y*,
that encodes dopachrome conversion enzyme (DCE), participates in both pathways to oxidate the DOPA and Dopamine precursors, generating the dark melanin pigment within the cuticle [5,11–15]. The Dopamine precursor is also used by N-beta-analylodopamine hydroxilase (NBAD) to generate yellow/tan colored sclerotin pigment. The last branch requires dopamine N-acetyl transferases (NAT) to convert Dopamine to N-acetyl dopamine (NADA), which is the precursor of unpigmented NADA sclerotin (Shamim et al, 2014). Phenoloxidases (POs) participate in all branches of the pigmentation pathway. The tan, ebony and black genes reported in Drosophila [16,17], the silkworm Bombyx mori [13], the butterfly Vanessa cardui [15] and the beetle Tribolium castaneum [18], encode NBAD pathway enzymes. aaNAT, with reported roles in pigment pattern of O. fasciatus [7] and Bombyx mori [19], encodes the central enzyme that generates unpigmented NADA sclerotin (arylalkylamine-N-acetyltransferase) (Fig 1A).

**Fig 1. Schematics of pigmentation pathways and Rhodnius prolixus loci associated to cuticle and eye color.** We have investigated cuticle pigmentation and eye color phenotypes resulting from knockdown for loci associated to: (A) the tyrosine pathway, involved in cuticle pigmentation in insects, and (B) pathways associated to the generation of ommochrome and pteridin pigments in the eye. In italic: red represents loci that resulted in a change in color compared to wild-type; pink indicates loci that resulted in no visible effect upon knockdown; black refers to loci that were investigated by others. PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase;
In insects, eye color requires the synthesis of ommochrome and pteridine pigments from tryptophan and guanine precursors, respectively, and their uptake into pigment granules by ABC transporters [10,20] (Fig 1B). *vermillon* (*v*), that encodes tryptophan oxidase, and *cinnabar* (*cn, or kynu*), that encodes kynurenin hydroxylase, are associated with the production of ommochromes [21–24]. Loss-of-function mutants for *sepia* (*se*), that encodes a glutathione-S-transferase, are characterized by loss of the bright red eye color in *D. melanogaster* due to a decrease in red pteridins [25]. In *D. melanogaster* [26] and *Tribolium castaneum* [27] *w, scarlet* (*st*) and *brown* (*bw*) loci encode ABC transporters that for heteromeric channels: proteins encoded by *bw* and *w* transport the red pteridines into cells of developing eyes, while *w* plus *st* transport brown ommochrome pigments. Eye color mutations affecting components of either pathway have been described in several insect orders [9,27–34].

The cuticle pigment pathway is also important for melanization associated with the immune response [35,36] and for detoxification of tyrosine ingested from diet [37], which is of particular importance for insects feeding on blood. Due to the excessive amount of protein ingested during the blood meal, mounting to several times their body weight, insects depend on the detoxification of dietary tyrosine for optimal fitness. Therefore, it has been suggested that blood-feeding insects evolved strategies to reduce the redox stress employing the tyrosine pathway [38]. Thus, investigating
how the melanin pathway was coopted to serve both for body color and a detox functions in blood feeding species may be particularly relevant for the biology of blood-sucking insects. A similar problem arises from the high levels of tryptophan present in the blood meal, also an essential precursor for ommochrome synthesis. Accordingly, the midgut of *R. prolixus* expresses high levels of both tryptophan and tyrosine degradation pathway enzymes after a blood meal [39].

The identification of loci that control pigmentation has lead to great advances in the development of transgenic strategies in insects. For instance, the identification and cloning of *D. melanogaster* *w*, and of *rosy*, that codes for the xanthine dehydrogenase required for pteridin synthesis in the eye, and the establishment of *w*- and *rosy*- mutant lines granted the development of transposable element-based transformation [40]. A decade later, identification of the *Ceratitis capitata* *w* gene enabled the development of germline transformation protocols in the medfly [41,42]. Visible markers were also decisive for modern CRISPR-based genome edition. In the first report that proved the principal of auto-replicative CRISPR-based gene drive elements in *D. melanogaster*, *y* was used as target for gene disruption [43]. Similarly, mosquito *kynurenine hydroxilase* was used to reveal the feasibility of gene drive in *Anopheles stephensis* [44]. Notably, in the context of gene drive lineage production, pigmentation genes may both serve as visible markers carried on transgenic constructs as well as markers for gene disruption, when used as target sites for the incorporation of gene drive constructs.

As kissing bugs play a major role in transmission of parasitic Chagas disease, identifying cuticle and eye color loci will aid in defining visible markers for future
transgenic and gene edition protocols. We hereby describe the identification and functional analyses of pigmentation pathway genes in *R. prolixus*. We show that knockdown of the *R. prolixus* orthologs of the *st* and *aaNAT* genes produce striking cuticle and eye color phenotypes. Remarkably, downregulation of these genes by RNA interference (RNAi) does not affect animal viability and female fertility in laboratory conditions. We demonstrate that several genes belonging to the cuticle color module display a conserved function in this insect, while one specific branch of the pathway, namely the NBAD tanning branch, appears to have lost most of its role in cuticle coloration. Our data shed light on the evolution of the pigmentation pathway in insects and provide easily scorable phenotypic markers, which will facilitate transgenesis, genome edition and, ultimately, the development of *R. prolixus* population control strategies.

**Results**

**Identification of pigmentation genes in *R. prolixus***

We have identified several putative cuticle and eye pigmentation genes in the *R. prolixus* genome, based on protein sequence similarity to *D. melanogaster*, *O. fasciatus* and *Anopheles sp.* One clear ortholog was identified for each of the most evolutionarily conserved genes, particularly those coding for enzymes of the tyrosine, ommochrome and pteridine synthesis pathways (Table 1; S1 Table). The expression for many of these genes was confirmed by performing searches in transcriptomic datasets (data not shown). Differently, for the identification of *y* as well as of genes
encoding putative ABC transporters, phylogenetic analyses were performed to identify the most likely orthologs among several sequences (S1 and S2 Fig). In agreement with the diversity of y paralogs reported in different insect species, we identified four y loci, three clear orthologs of D. melanogaster y, y B and y C. The forth y, which we termed yellow-like (y-like), probably diverged from the main y branch. For eye pigment ABC transporters, we found that R. prolixus presents one w, one st, and two bw orthologs, which we refer to as bw A and bw B. This duplication event might be unique to R. prolixus, since only one w, one st and one br gene have been described in D. melanogaster, T. castaneum [27], and B. mori [34]. Notably, the analysis presented below shows that most of the genes herein identified are functional, confirming our in silico identification.

Table 1. Pigmentation-associated genes identified and functionally analyzed in this study. Genes were identified by sequence similarity to D. melanogaster and Anopheles sp. genes and validated by phylogenetic analysis. Gene names were defined following terminology used for other insects, mostly based on the loss-of-function phenotype.

Visible phenotypes associated with loss of function for genes in the melanin synthesis pathway

To identify suitable loci as visible markers for transgenesis, we initially investigated the effect of tyrosine pathway genes on viability of the progeny and putative changes in cuticle pigmentation (Fig. 2; S2 Table). To this aim we performed
parental RNAi (pRNAi) by injecting double-stranded RNA (dsRNA) molecules specific to each cognate gene into the hemocoel of adult females. A decrease in adult or embryo viability upon gene knockdown (KD) would exclude the gene as a good landing site for transgenesis. This was particularly true for DDC, even though the few surviving DDC KD embryos displayed total loss of pigmentation, as previously reported (S2 Table; [45]). On the other hand, y KD (RPRC005424) displayed a change in cuticle coloration in the thorax, head and legs, with no significant decrease in viability (Fig 2C,D). Additional tyrosine pathway loci presented no effect on pigmentation by pRNAi. We also investigated whether the blood diet had any effect on animals resulting from pRNAi. We observed no effect beyond those already present at nymph eclosion.

Table 2. General phenotypic effects of tyrosine, ommochrome and pteridine pathway gene knockdowns

Fig 2. Parental knockdown for a limited set of putative R. prolixus tyrosine pathway loci affects embryo and first instar viability. We performed pRNAi for tyrosine pathway loci and analyzed their effect on injected animals and their progeny. A) Injection protocol for pRNAi. B) Viable progeny resulting from dsRNA injected females. C,D) First instar nymphs resulting from females injected with control (C) or y (D) dsRNA, showing that y KD animals have a slightly lighter cuticle, especially visible in the thorax and legs (asterisks). E) Molting rate to second instar of first instar nymph offspring from dsRNA injected females, after a blood meal. Only animals from y C
dsRNA-injected females showed significant loss in viability. Numbers displayed inside bars correspond to individual eggs (B) or first instar nymphs (E) analyzed.

Next, we assayed tyrosine pathway gene function by injecting dsRNA in fifth instar nymphs. After feeding, these nymphs molt in around 16 days as unpigmented adults, regaining full color in approximately 12 hours. In this condition we observed a significant change in cuticle color for y and aaNAT KD (Fig 3). This is especially evident in the thorax, where three different colored stripes are seen: dark stripes which we called black (bl), brown stripes we termed tan (tan), and clear stripes identified as white (wh). In y KD the bl stripes are lighter, consistent with a role for y in generating black/brown melanin (Fig 3C). Two other yellow loci, y C (RPRC008209) and y-like (RPRC014337) displayed only a weak effect on cuticle pigmentation. However, the y C plus y-like double KD resembles the y KD cuticles, suggesting redundancy among yellow paralogs (S3 Fig). On the other hand, aaNAT KD cuticles display a striking phenotype: they are homogeneously dark, losing the tan and wh stripes of the thorax and any pattern characteristic of the insect throughout the entire body (Fig 3E). Since aaNAT is predicted to generate uncolored sclerotin, this observation indicates that aaNAT function is required to produce all the light color patterns of the insect body, including the tan and wh stripes of the thorax. Such effect is surprising given the weak effect of aaNAT loss-of-function shown in other insects, where only a few dark spots are gained [7,19]. Equally surprising is the fact that ebony (e) and tan (t) KD had no effect on pigmentation, given the extremely dark loss-of-function phenotype observed in several insect species (S2 Table;
[7,13,15,46]). Unfortunately, we were unable to obtain a *black* cDNA for dsRNA production and functional analysis using ovaries or embryos. Low expression levels in the gut and early embryogenesis suggest, however, that *black* is functional in other tissues. Due to the strong phenotype displayed by *aanAT* KD, we henceforth refer to the locus associated to this function as *preto* (*pro*), the Brazilian portuguese term for "black".

**Fig 3. Cuticle pigmentation phenotypes resulting from knockdown of the tyrosine pathway genes.** A) RNAi strategy: fifth instar nymphs were injected with dsRNA specific to tyrosine pathway genes and the phenotype was analyzed in the adult. B) Viability of animals injected with dsRNA, after a blood meal. No significant difference between experimental conditions versus control was observed Numbers in histograms correspond to the number of fifth instar nymphs injected. C-F) Control (C,C') and cuticle phenotypes resulting from knockdown for *y* (D,D') *bw A* (E,E'), and *aanAT/pro* (F,F'). C'-F') Higher magnification of C-F showing details of the three-color pattern of the *R. prolixus* first thoracic segment in control and knockdown animals.

To further investigate the effect of different melanin pathway branches on cuticle pigmentation, we performed double knockdowns for *e* plus *y* as well as for *pro* plus *y* (Fig 4). Double KD for *e* and *y* produced a phenotype comparable to *y* KD alone, both with respect to head, thorax and abdomen patterns as well as to wing pigmentation (Fig 4A-C, G-J). Together with the single KD assays reported above, this observation supports the conclusion that the NBAD branch does not control
cuticle pigmentation in *R. prolixus*. On the other hand, *pro* plus *y* double KD generates animals that are unpatterned and light brown up to 24h after molting (Fig 4D-F, K-L), when control insects are already fully pigmented. Subsequently, these animals gain a dark cuticle that resembles the *pro* KD phenotype. This delayed pigmentation pattern observed in the double *y* + *pro* KD could be explained by a decrease in enzymatic activity in both the NADA (*pro*) and brown/black melanin (*y*) branch, with residual Yellow activity slowly building up the dark pigmentation.

**Fig 4. Double knockdown for distinct tyrosine pathway branches points to the absence of a cuticle pigmentation function for the NBAD branch.** Effects on body (A-F) and wing (G-L) pigmentation, resulting from single or double KD for tyrosine pathway loci. A,H) e KD cuticles are identical to wild-type or control (G); B,I) y KD; C,J) e + y KD; D,K) pro KD; E) y + pro KD at 24 hours after moulting; F,L) y + pro KD at 48h hours after moulting. G) Wings from control KD.

**Loss of function for classical eye color genes generates visible phenotypes**

*Anopheles stephensi kynu*, the ortholog of *D. melanogaster cn*, has been successfully used as a phenotypic marker in previous transgenic studies, since loss of *kynu* function generates white eyes [44,47]. In order to investigate the function of the *R. prolixus cn/kynu* ortholog, we injected dsRNA in fifth instar nymphs, and looked for a visible eye color phenotype in the adults that emerge after molting. As a result, *cn* KD adult *R. prolixus* have reddish eyes (Fig 5A,B,D). pRNAi results in progeny displaying a similar eye color phenotype. These first instar nymphs have
either reddish eyes or display a red circle around the black colored eye (Fig 5F,G,H). Interestingly, this pattern is observed in early molting wild-type animals where the wild-type black color slowly builds from a red eye, but, unlike cn KD, the red circle disappears with age (S4 Fig). Importantly, we observed no effect of cn KD on viability of the egg/first instar nymph before a blood meal. However, feeding cn KD first instars with a blood meal resulted in death of all animals before they could molt to second instars (Fig 5J). This suggests that cn function is essential for the molting process.

Fig. 5. Phenotypes resulting from the knockdown of genes involved in the production and transport of eye pigments. A) Adult viability and B-E) Adult eye color phenotypes observed following fifth instar dsRNA injections for B) control, C) bw A, D) cn and E) st. Numbers in histograms correspond to the number of fifth instar nymphs injected. F-I) First instar phenotypes resulting from adult female dsRNA injections. F) Egg viability resulting from pRNAi. G-I) First instar eye color phenotypes observed following pRNAi for G,G') control, H,H') cn and I,I) st. J) Molting rate to second instar of first instar nymph offspring from dsRNA injected females after a blood meal. The number of individual first instar nymphs analyzed is displayed inside bars. Note that after blood ingestion none of the cn KD animals survived.

Next, we performed KD for putative ABC pigment transporters. Surprisingly, the KD of the w and bw B orthologs in R. prolixus did not produce obvious phenotypes (Supp Table II), although red pigment spots in adult connexives are lost
in bw B KD (Fig S3). Conversely, KDs for st and bw A showed striking effects (Fig 5): st KD gives rise to red eyes, suggesting that st acts in the eye for the transport of dark ommochrome pigments (Fig 5E,G,I). st KD resulting from dsRNA injection in fifth instar nymphs also alters cuticle coloration in addition to generating a red eye phenotype. Cuticles are reddish in appearance, missing the wh stripes of the head and body (Fig 5B,E). On the other hand, bw A has no effect on the eye, but shows a clear effect on the cuticle (Fig 5B,C). In the animal's thorax, originally light regions of the cuticle are now tainted red as shown for st KD (Fig 5C and 3E). Interestingly, KD of se, whose protein product has been reported to function in the synthesis of pteridines in several insect species, generates a thoracic pigmentation phenotype that is identical to bw A KD (S3 Fig). This suggests that bw A and se KD result in loss of a pigment that acts to mask the red cuticle color. Alternatively, red pteridines are transported away from the cuticle or modified to another color in order to maintain light stripes and patterns.

Discussion

An alternative strategy for cuticle pigmentation in R. prolixus

We have analyzed several R. prolixus loci coding for enzymes of the tyrosine pathway, which have been classically associated to the production of cuticle color. Genetic loci that encode enzymes in the initial steps of the tyrosine pathway frequently produce changes in cuticle coloration as well as cuticle integrity/strength, consistent with branching of the pathway for the production of melanin and sclerotin. Accordingly,
knockdowns and loss-of-function mutants for the genes encoding the initial enzymes of the pathway PAH, TH and DDC lead to loss of cuticle coloration in *D. melanogaster*, *O. fasciatus* and *V. cardui*, to soft cuticles in *T. castaneum* and *Anopheles sinenses* [14,48], and decrease in first instar hatching in *R. prolixus* and *B. mori* [38,49]. The reduction in hatching rates may result from softness of the eggshell, as eggshell structure depends on adequate sclerotization, making it difficult for the emerging nymphs to punch through the eggshell. Furthermore, pigmentation may be associated to desiccation resistance as shown for *Aedes*, *Anopheles* and *Culex* [50].

The function of phenoloxidases (PO), which participate in all melanin and sclerotin branches, was previously analyzed in *R. prolixus* by gene knockdown and did not produce apparent phenotypes [37]. However, due to the large number of PO coding genes (at least four) in the *R. prolixus* genome, this may result from functional redundancy. Subsequent steps of the tyrosine pathway are restricted either to the melanin or sclerotin production pathways, and are thus more likely to contribute useful phenotypic markers. Among the putative loci tested, *y* produced the expected yellow loss-of-function cuticle phenotype, where dark black stripes of the adult thorax are lightened and wings lose their dark coloration. This is consistent with a role in generating brown/black melanin. Furthermore, the strong effect of the double *y C plus y-like* KD, as compared to single KDs, suggests functional redundancy of *yellow* loci. Phylogenetic analysis of *R. prolixus yellow* genes conforms to this view (S2 Fig).

Using Dopamine as substrate, the NBAD sclerotin branch has been associated to the generation of tanned/yellow cuticle. In *Drosophila*, *Oncopeltus*, *Tribolium*, *Bombyx* and *V. cardui*, *e (ebony)* and *bl (black)* KDs produce animals with dark cuticle
due to loss of dopamine conversion to the tan/yellow pigment, leaving Dopamine available for the conversion to black pigment [7,13,15,46,51]. Conversely, loss of t (tan) function results in light pigmented cuticles in Drosophila, O. fasciatus, N. lugens and C. capitata [7,46,52]. The functions of the e and t genes in blood feeding insects has not been investigated yet, although a study in A. gambiae showed that a bl allele generates animals with dark cuticle and reduced fertility and vigor [53]. Here we show that R. prolixus e and t KD do not produce a visible phenotype, although a small reduction in insect viability was observed in both assays and mRNA levels clearly reduce (S5 Fig). NBAD synthase and NBAD hydrolase, encoded by e and t, respectively, have been implicated in several different processes. They exert a role in brain neurotransmitter metabolism [52], and e is expressed in the foregut and tracheal epidermis in D. melanogaster [51]. Their effect on tyrosine detoxification, which could also explain the loss of viability, has not been explored.

Surprisingly, inhibiting the NADA branch by aaNAT/pro KD results in a much stronger phenotype than shown for other insects [7,19]. The dramatic effect of pro KD on cuticle pigmentation in R. prolixus suggests that AaNAT activity is required to produce low-pigmented areas and thus generate specific cuticle patterns by "erasing" color. Taking into account the lack of any visible effect of e and t KDs, this suggests that R. prolixus evolved the preferential use of non-pigmented NADA sclerotin and dark melanin as pigments for cuticle coloration pattern, with little or no contribution from the NBAD synthesis enzymes (see Fig. 1). Recently, it was shown that aaNAT RNAi in the black colored Platymeris biguttatus bug obliterates white spots as well as yellow and red colors. Together with aaNAT/pro KD phenotype in R. prolixus, these
findings may suggest that hemimetabolous blood feeding insects rely greatly on the NADA branch for cuticle color patterning.

Notably, kissing bugs display dark and frequently monotonous pigmentation patterns, as compared to their plant feeding relatives [54]. Among blood feeding Triatomines, the sole color that diverges from the black-tan-clear pallet is red. Interestingly, the red color originates in *R. prolixus* head and thorax as a result of *st*, *bw A* and *se* KDs (Fig 3E, Fig 5C and E; S3D Fig), resembling some *Triatoma* and *Panstrongylus* species cuticle pattern. Differently, *bw B* KD results in loss of red pigments that are brought through small veins that connect to the abdominal connexivum (S3F Fig). This indicates that red pigments, unrelated to the melanin pathway, are transported by ABC proteins to define the color of the kissing bug cuticle.

In butterflies, the classical eye color-associated genes *v*, *cn*, and *w* are required for pigment patterning during wing development [55]. Importantly, ommochromes, pteridines and ABC transporters regulate cuticle pigmentation in many insect orders [10]. It will be interesting to investigate whether kissing bugs in general use a limited set of tyrosine pathway genes in addition to pteridine and ommochrome pigments for cuticle pigmentation as our results suggest for *R. prolixus*.

**Pigment transporters in *R. prolixus***

Loss-of-function of *w* genes generates eye pigmentation phenotypes in most insects studied to date. However, we observed no visible phenotype upon *R. prolixus w* KD. KD of *st*, on the other hand, generated red eyes and red tainted cuticle in the head and thorax. The red eye phenotype is likely due to a reduction in the transport of
black ommochrome pigments, since KD for cn, that encodes an enzyme at the basis of the ommochrome synthesis pathway, generates a similar phenotype. An open question thus concerns the identity of the gene coding the ABC transporter that combines with the st product to form heterodimeric channels in the eye pigment granules, since we observed no eye pigmentation phenotype in KDs for other ABC transporters herein analyzed. In the head and thorax, St and Bw A likely interact to transport pteridine pigments, since st, bw A and se KDs display a similar phenotype. Notably, bw B KD results in loss of spatially restricted abdominal red spots, a phenotype only mirrored in the cn KD. Thus bw B probably transports ommochrome pigments in the abdomen. These results also suggest that bw A and bw B may have spatially distinct expression patterns, and transport different pigments in the head and thorax versus abdomen. In addition, bw B probably forms heterodimers with a different ABC protein than st, since st KD does not change the abdominal pattern. The lack of an eye color phenotype upon w, bw A and bw B KD is surprising, given the classical phenotypes reported for D. melanogaster. However, knockdown of a T. castaneum brown ortholog indicated no function in eye either [27]. Future studies directed to investigate functional redundancies in the genes coding for ABC transporters in R. prolixus may shed light on the control of eye pigmentation in this species.

**Visible markers for construct integration in R. prolixus**

Using knockdown assays we have identified several loci that change eye or cuticle color and thus may serve as transformation markers in transgenic constructs or markers for construct integration upon CRISPR-based genome edition and HDR. Our
results show that loss-of-function for a few loci generates visible phenotypes while having little effect on viability/fertility. Considering that the knockdown approximates the effect of gene disruption, these loci will certainly be useful as target integration sites. The most promising in this sense are aaNAT/pro and st, which present easily scored phenotypes and no significant effect on viability. se and bw A also share similar characteristics, although the red coloration is harder to perceive at a glance.

In contrast to the loci above, y C KD led to a small drop in nymph viability after blood feeding. This effect may result from loss of a waterproofing function, as shown for the beetle T. castaneum [56]. Therefore, despite the clear cuticle phenotype, apparent redundancy among R. prolixus y loci and broad use as marker in several insect species, we consider its potential as target integration site smaller than pro and st. Hence, our analysis has pointed out specific loci as markers for use in transgenic studies and genome edition in the kissing bug model Rhodnius prolixus.
Materials and Methods

Insect rearing

*R. prolixus* rearing was performed at 28°C and 70-75% humidity. Animal care and experimental protocols were conducted following guidelines of the Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro (CAUAP-UFRJ) and the NIH Guide for Care and Use of Laboratory Animals (ISBN0-309-05377-3). Technicians dedicated to the animal facility at the Institute of Medical Biochemistry (UFRJ) conducted all aspects related to rabbit husbandry under strict guidelines to ensure careful and consistent animal handling.

Identification of cuticle and eye color related genes in *R. prolixus* genome and phylogenetic construction

*D. melanogaster* and *Anopheles stephensi* protein sequences were used as query to BLAST into the *Rhodnius prolixus* genome (https://www.vectorbase.org/). After manual curation, protein sequences were aligned using the CLUSTALW algorithm available at the MEGA6 package [57]. Accession numbers for the genes analyzed are provided in Table 1. For phylogenetic analysis of ABC transporter and yellow genes, the evolutionary histories were inferred applying a Maximum Likelihood method [58] as described in Brito et al (2018)[59]. Briefly, the amino acid sequences were aligned by the Multiple Sequence Alignment with Log Expectation (MUSCLE, version 3.8.31) method [60], employing standard parameters. The evolutionary history was inferred by Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) [61], and visualized using interactive Tree of Life (iTOL, v2)
The tree was validated by 500 bootstraps replications. All values higher of bootstraps were indicated in nodes. The amino acid sequences of proteins used in this study were obtained from VectorBase (https://www.vectorbase.org/), FlyBase (http://flybase.org/) and NCBI (https://www.ncbi.nlm.nih.gov) the identification of each protein is indicated in the tree.

RNA interference assays (RNAi)

Double stranded RNA was synthesized from PCR products containing T7 promoter sequences at both ends as previously described [63]. Two successive PCRs were performed, the first to amplify the open reading frame of the gene of interest and the second added T7 promoter sequences at both ends. Primer pairs used in the first PCR are listed in Supp Table I. In vitro transcription was performed with Megascript kit (Ambion) as per manufacturer instructions. Two microliters of each dsRNA (1 μg/μl) was injected in the abdomen of adult females three to five days prior blood feeding. Eggs were collected, counted, and the hatch rate defined after 20 days at 28°C. For fifth instar RNAi, 2 microliters dsRNA (1 μg/μl) were injected into female or male abdomen. The insects were blood fed 5 days after the injection and let develop to the adult stage.

Total RNA extraction and RT-PCR assays

For cDNA generation total RNA was extracted from eggs, first instar nymphs and carcasses using Trizol Reagent (Invitrogen) as per manufacturer instructions. Total RNA was treated with Turbo DNA Free (Ambion) to remove genomic DNA
traces. The resulting DNA-free total RNA was subjected to in vitro Reverse Transcription (RT) with Superscript III (Invitrogen). 1µg of total RNA was used for each reaction and assays were conducted in biological triplicates. The oligonucleotides used in RT-PCR assays are listed in Suppl. Table I.

**Image processing**

Microscopic images were obtained using a Leica Stereomicroscope, always on live animals. To minimize possible variation of the captured images, the background microscope and camera settings, as well image processing were standardized. To avoid cuticle pigmentation age related changes, all images were acquired during equivalent periods after molting. Adults were imaged 5 days after molt and first instars were imaged 2 days after eclosion.

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1. Wittkopp PJ, Beldade P. Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. Semin Cell Dev Biol. 2009;20: 65–71.

2. Morgan TH. SEX LIMITED INHERITANCE IN DROSOPHILA. Science. 1910;32: 120–122.

3. Lewis EB. The Pseudoalleleism of White and Apricot in Drosophila Melanogaster. Proc Natl Acad Sci U S A. 1952;38: 953–961.

4. Chia W, Howes G, Martin M, Meng YB, Moses K, Tsubota S. Molecular analysis of the yellow locus of Drosophila. EMBO J. 1986;5: 3597–3605.

5. Wittkopp PJ, True JR, Carroll SB. Reciprocal functions of the Drosophila yellow and ebony proteins in the development and evolution of pigment patterns. Development. 2002;129: 1849–1858.

6. Liu J, Lemonds TR, Popadić A. The genetic control of aposematic black pigmentation in hemimetabolous insects: insights from Oncopeltus fasciatus. Evol Dev. 2014;16: 270–277.

7. Liu J, Lemonds TR, Marden JH, Popadić A. A Pathway Analysis of Melanin Patterning in a Hemimetabolous Insect. Genetics. 2016. pp. 403–413. doi:10.1534/genetics.115.186684

8. Zhang L, Wang M-Y, Li X-P, Wang X-T, Jia C-L, Yang X-Z, et al. A small set of differentially expressed genes was associated with two color morphs in natural populations of the pea aphid Acyrthosiphon pisum. Gene. 2018;651: 23–32.

9. Xue W-H, Xu N, Yuan X-B, Chen H-H, Zhang J-L, Fu S-J, et al. CRISPR/Cas9-mediated knockout of two eye pigmentation genes in the brown planthopper, Nilaparvata lugens (Hemiptera: Delphacidae). Insect Biochem Mol Biol. 2018;93: 19–26.

10. Shamim G, Ranjan SK, Pandey DM, Ramani R. Biochemistry and biosynthesis of insect pigments. European Journal of Entomology. 2014. pp. 149–164. doi:10.14411/eje.2014.021

11. Walter MF, Black BC, Afshar G, Kermabon A-Y, Wright TRF, Biessmann H. Temporal and spatial expression of the yellow gene in correlation with cuticle formation and DOPA decarboxylase activity in drosophila development. Developmental Biology. 1991. pp. 32–45. doi:10.1016/s0012-1606(05)80005-3

12. Neckameyer WS, White K. Drosophila tyrosine hydroxylase is encoded by the pale locus. J Neurogenet. 1993;8: 189–199.

13. Futahashi R, Sato J, Meng Y, Okamoto S, Daimon T, Yamamoto K, et al. yellow and ebony are the responsible genes for the larval color mutants of the silkworm Bombyx mori. Genetics. 2008;180: 1995–2005.
14. Gorman MJ, Arakane Y. Tyrosine hydroxylase is required for cuticle sclerotization and pigmentation in Tribolium castaneum. Insect Biochem Mol Biol. 2010;40: 267–273.
15. Zhang L, Martin A, Perry MW, van der Burg KRL, Matsuoka Y, Monteiro A, et al. Genetic Basis of Melanin Pigmentation in Butterfly Wings. Genetics. 2017;205: 1537–1550.
16. Hovemann BT, Ryseck RP, Walldorf U, Störtkuhl KF, Dietzel ID, Dessen E. The Drosophila ebony gene is closely related to microbial peptide synthetases and shows specific cuticle and nervous system expression. Gene. 1998;221: 1–9.
17. True JR, Yeh S-D, Hovemann BT, Kemme T, Meinertzhagen IA, Edwards TN, et al. Drosophila tan encodes a novel hydrolase required in pigmentation and vision. PLoS Genet. 2005;1: e63.
18. Arakane Y, Lomakin J, Beeman RW, Muthukrishnan S, Gehrke SH, Kanost MR, et al. Molecular and functional analyses of amino acid decarboxylases involved in cuticle tanning in Tribolium castaneum. J Biol Chem. 2009;284: 16584–16594.
19. Zhan S, Guo Q, Li M, Li M, Li J, Miao X, et al. Disruption of an N-acetyltransferase gene in the silkworm reveals a novel role in pigmentation. Development. 2010;137: 4083–4090.
20. Figon F, Casas J. Ommochromes in invertebrates: biochemistry and cell biology. Biol Rev Camb Philos Soc. 2018. doi:10.1111/brv.12441
21. Sullivan DT, Kitos RJ, Sullivan MC. Developmental and genetic studies on kynurenine hydroxylase from Drosophila melanogaster. Genetics. 1973;75: 651–661.
22. Paton DR, Sullivan DT. Mutagenesis at the cinnabar locus in Drosophila melanogaster. Biochem Genet. 1978;16: 855–865.
23. Lorenzen MD, Brown SJ, Denell RE, Beeman RW. Cloning and characterization of the Tribolium castaneum eye-color genes encoding tryptophan oxygenase and kynurenine 3-monooxygenase. Genetics. 2002;160: 225–234.
24. Han Q, Beerntsen BT, Li J. The tryptophan oxidation pathway in mosquitoes with emphasis on xanthurenic acid biosynthesis. J Insect Physiol. 2007;53: 254–263.
25. Kim J, Suh H, Kim S, Kim K, Ahn C, Yim J. Identification and characteristics of the structural gene for the Drosophila eye colour mutant sepia, encoding PDA synthase, a member of the omega class glutathione S-transferases. Biochem J. 2006;398: 451–460.
26. Ewart GD, Howells AJ. ABC transporters involved in transport of eye pigment precursors in Drosophila melanogaster. Methods Enzymol. 1998;292: 213–224.
27. Grubbs N, Haas S, Beeman RW, Lorenzen MD. The ABCs of eye color in Tribolium castaneum: orthologs of the Drosophila white, scarlet, and brown Genes. Genetics. 2015;199: 749–759.
28. Besansky NJ, Bedell JA, Benedict MQ, Mukabayire O, Hilfiker D, Collins FH. Cloning and characterization of the white gene from Anopheles gambiae. Insect Mol Biol. 1995;4: 217–231.
29. Li J. Oxidation of 3-hydroxykynurenine to produce xanthommatin for eye pigmentation: a
30. Rasgon JL, Scott TW. Crimson: A Novel Sex-Linked Eye Color Mutant of Culex Pipiens L. (Diptera: Culicidae). Journal of Medical Entomology. 2004. pp. 385–391. doi:10.1603/0022-2585-41.3.385

31. Insausti TC, Le Gall M, Lazzari CR. Oxidative stress, photodamage and the role of screening pigments in insect eyes. J Exp Biol. 2013;216: 3200–3207.

32. Khan SA, Reichelt M, Heckel DG. Functional analysis of the ABCs of eye color in Helicoverpa armigera with CRISPR/Cas9-induced mutations. Sci Rep. 2017;7: 40025.

33. Jiang Y, Lin X. Role of ABC transporters White, Scarlet and Brown in brown planthopper eye pigmentation. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2018. pp. 1–10. doi:10.1016/j.cbpb.2018.04.003

34. Zhang H, Kiuchi T, Hirayama C, Katsuma S, Shimada T. Bombyx ortholog of the Drosophila eye color gene brown controls riboflavin transport in Malpighian tubules. Insect Biochemistry and Molecular Biology. 2018. pp. 65–72. doi:10.1016/j.ibmb.2017.11.012

35. Whitten MMA, Coates CJ. Re-evaluation of insect melanogenesis research: Views from the dark side. Pigment Cell Melanoma Res. 2017;30: 386–401.

36. Bilandžija H, Laslo M, Porter ML, Fong DW. Melanization in response to wounding is ancestral in arthropods and conserved in albino cave species. Sci Rep. 2017;7: 17148.

37. Sterkel M, Perdomo HD, Guizzo MG, Barletta ABF, Nunes RD, Dias FA, et al. Tyrosine Detoxification Is an Essential Trait in the Life History of Blood-Feeding Arthropods. Curr Biol. 2016;26: 2188–2193.

38. Sterkel M, Oliveira JHM, Bottino-Rojas V, Paiva-Silva GO, Oliveira PL. The Dose Makes the Poison: Nutritional Overload Determines the Life Traits of Blood-Feeding Arthropods. Trends Parasitol. 2017;33: 633–644.

39. Ribeiro JMC, Genta FA, Sorgine MHF, Logullo R, Mesquita RD, Paiva-Silva GO, et al. An insight into the transcriptome of the digestive tract of the bloodsucking bug, Rhodnius prolixus. PLoS Negl Trop Dis. 2014;8: e2594.

40. Rubin GM, Spradling AC. Genetic transformation of Drosophila with transposable element vectors. Science. 1982;218: 348–353.

41. Loukeris TG, Livadaras I, Arcà B, Zabalou S, Savakis C. Gene transfer into the medfly, Ceratitis capitata, with a Drosophila hydei transposable element. Science. 1995;270: 2002–2005.

42. Zwiebel LJ, Saccone G, Zacharopoulou A, Besansky NJ, Favia G, Collins FH, et al. The white gene of Ceratitis capitata: a phenotypic marker for germline transformation. Science. 1995;270: 2005–2008.

43. Gantz VM, Bier E. Genome editing. The mutagenic chain reaction: a method for
converting heterozygous to homozygous mutations. Science. 2015;348: 442–444.

44. Gantz VM, Jasinskiene N, Tatarenkova O, Fazekas A, Macias VM, Bier E, et al. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. Proc Natl Acad Sci U S A. 2015;112: E6736–43.

45. Sterkel M, Ons S, Oliveira PL. DOPA decarboxylase is essential for cuticle tanning in Rhodnius prolixus (Hemiptera: Reduviidae), affecting ecdysis, survival and reproduction. Insect Biochem Mol Biol. 2019;108: 24–31.

46. Futahashi R, Banno Y, Fujiwara H. Caterpillar color patterns are determined by a two-phase melanin gene prepatternning process: new evidence from tan and laccase2. Evol Dev. 2010;12: 157–167.

47. Han Q, Calvo E, Marinotti O, Fang J, Rizzi M, James AA, et al. Analysis of the wild-type and mutant genes encoding the enzyme kynurenine monoxygenase of the yellow fever mosquito, Aedes aegypti. Insect Mol Biol. 2003;12: 483–490.

48. True JR, Edwards KA, Yamamoto D, Carroll SB. Drosophila wing melanin patterns form by vein-dependent elaboration of enzymatic prepatterns. Curr Biol. 1999;9: 1382–1391.

49. Liu C, Yamamoto K, Cheng T-C, Kadono-Okuda K, Narukawa J, Liu S-P, et al. Repression of tyrosine hydroxylase is responsible for the sex-linked chocolate mutation of the silkworm, Bombyx mori. Proc Natl Acad Sci U S A. 2010;107: 12980–12985.

50. Farnesi LC, Vargas HCM, Valle D, Rezende GL. Darker eggs of mosquitoes resist more to dry conditions: Melanin enhances serosal cuticle contribution in egg resistance to desiccation in Aedes, Anopheles and Culex vectors. PLoS Negl Trop Dis. 2017;11: e0006063.

51. Pérez MM, Schachter J, Berni J, Quesada-Allué LA. The enzyme NBAD-synthase plays diverse roles during the life cycle of Drosophila melanogaster. J Insect Physiol. 2010;56: 8–13.

52. Pérez MM, Sabio G, Badaracco A, Quesada-Allué LA. Constitutive expression and enzymatic activity of Tan protein in brain and epidermis of Ceratitis capitata and of Drosophila melanogaster wild-type and tan mutants. Insect Biochem Mol Biol. 2011;41: 653–659.

53. Benedict MQ, McNitt LM, Cornel AJ, Collins FH. A new marker, black, a useful recombination suppressor, ln(2)2, and a balanced lethal for chromosome 2 of the mosquito Anopheles gambiae. Am J Trop Med Hyg. 1999;61: 618–624.

54. Jurberg J, Galvão C, Weirauch C, Moreira FFF. Hematophagous Bugs (Reduviidae, Triatominae). True Bugs (Heteroptera) of the Neotropics. 2015. pp. 353–393. doi:10.1007/978-94-017-9861-7_13

55. Reed RD, Nagy LM. Evolutionary redeployment of a biosynthetic module: expression of eye pigment genes vermilion, cinnabar, and white in butterfly wing development. Evol Dev. 2005;7: 301–311.

56. Noh MY, Kramer KJ, Muthukrishnan S, Beeman RW, Kanost MR, Arakane Y. Loss of
function of the yellow-e gene causes dehydration-induced mortality of adult Tribolium castaneum. Dev Biol. 2015;399: 315–324.

57. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30: 2725–2729.

58. Hall BG. Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol. 2013;30: 1229–1235.

59. Brito T, Julio A, Berni M, de Castro Poncio L, Bernardes ES, Araujo H, et al. Transcriptomic and functional analyses of the piRNA pathway in the Chagas disease vector Rhodnius prolixus. PLoS Negl Trop Dis. 2018;12: e0006760.

60. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32: 1792–1797.

61. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol. 2018;35: 1547–1549.

62. Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res. 2016;44: W242–5.

63. Berni M, Fontenele MR, Tobias-Santos V, Caceres-Rodrigues A, Mury FB, Vionette-do-Amaral R, et al. Toll signals regulate dorsal-ventral patterning and anterior-posterior placement of the embryo in the hemipteran Rhodnius prolixus. Evodevo. 2014;5: 38.
Supporting Information Legends

**S1 Fig. Phylogenetic analysis of *R. prolixus yellow* gene family.** Genes were identified and names were given based on greatest similarity of *R. prolixus yellow* to previously characterized insect *yellow* genes.

**S2 Fig. Phylogenetic analysis of *R. prolixus* ABC family transporters.** A limited set of *R. prolixus* ABC transporters is displayed, particularly those that displayed highest similarity to genes associated to pigmentation phenotypes in other insects.

**S3 Fig. Cuticle pigmentation resulting from knockdown for additional tyrosine pathway and pteridine synthesis loci.** Fifth instar nymphs were injected with 2μg/ml dsRNA and the phenotype was analyzed in adults. A) Recently emerged wild type adult before pigment deposition in the cuticle. Eyes are already dark. (B-J) 76h old adults resulting from dsRNA injections against B) GFP; C) pugilist; D) sepia; E) cinnabar; F) brown B; G) tan; H) yellow C; I) yellow-like; J) yellow C-like plus yellow-like (1μg/ml each). Upper right insets show eye color phenotype in cinnabar KD (E) compared to control (B). Bottom right insets show details of connexive pigmentation, with loss of red pigment spots in cinnabar (E) and brown B (F), compared to control (B) KD. Note the red cuticle color in sepia KD and the light color in yellow C plus yellow-like double KD.
**S4 Fig. Temporal progression of eye and cuticle color.** Color upon emergence of first instar nymphs (top) and emergence of adult male and female *R. prolixus* (bottom). The progressive appearance of a clear red ring around the eye and darkening with early aging implies that effects on eye color should be considered only after a 76 hour period, after which eye color is constant. For adults, the black eye color is already evident upon emergence, different from the cuticle that is initially uncolored and reveals the underlying pink tissues, and progressively darkens to full color at 24 hours after emergence.

**S5 Fig. Extent of gene knockdown for loci associated to cuticle and eye color phenotypes.** qRT-PCR analysis of recently emerged adult cuticles from fifth instar nymphs injected with dsRNA for (A) yellow, (B) yellow-C, (C) ebony, (D) tan, and (E) scarlet. Despite no visible phenotype, the levels of *ebony* and *tan* mRNAs show a significant decrease compared to wild type.

**S1 Table. Loci investigated in this study, with accession numbers and primers used for RNA interference.**

**S2 Table. Consolidated effects of gene knockdown for tyrosine (top) and tryptophan (bottom) pathway associated loci.** "Unpigmented connexives" refers to lack of red pigment in veins that transport pigments to connexives (S3 Figure).
Figure 2

(B) Graph showing the number of eggs produced and the rate of eclosion for different treatments: DDC, pro, e, t, y, y-like, y C.

(E) Graph showing the percentage of second instar moulting rate for different treatments: ctrl, pro, yC, y, y-like.
| locus        | symbol | protein/enzyme encoded                          | process involved                              | accession #          |
|-------------|--------|------------------------------------------------|-----------------------------------------------|---------------------|
| DDC         | DDC    | Dopa decarboxylase                              | tyrosine pathway                              | RPRC005884-RA       |
| aaNAT/preto | pro    | Arylalkylamine-N-acetyltransferase              | tyrosine pathway/NADA branch                  | RPRC015310-RA       |
| ebony       | e      | N-beta-arylhydrazine synthase                   | tyrosine pathway/NBAD branch                  | RPRC007578-RA       |
| tan         | t      | N-beta-arylhydrazine hydroxylase                | tyrosine pathway/NBAD branch                  | RPRC007817-RA       |
| black       | bl     | Aminoacid decarboxylase                         | tyrosine pathway/NBAD branch                  | RPRC010142-RA       |
| yellow      | y      | Dopochrome conversion enzyme (DCE)             | tyrosine pathway/ melanin synthesis           | RPRC005424-RA       |
| yellow C    | y C    | "                                              | tyrosine pathway/ melanin synthesis           | RPRC008209-RA       |
| yellow-like | y-like | "                                              | tyrosine pathway/ melanin synthesis           | RPRC014337-RA       |
| sepia       | se     | Glutatione-S-transferase                        | pteridine synthesis                           | RPRC007741-RA       |
| white       | w      | ABC transporter                                 | eye pigment transporter                        | RPRC012709-RA       |
| scarlet     | st     | "                                              | eye pigment transporter                        | RPRC010854-RA       |
| brown A     | bw A   | "                                              | eye pigment transporter                        | RPRC009214-RA       |
| brown B     | bw B   | "                                              | eye pigment transporter                        | RPRC002598-RA       |
| cinnabar    | cn     | Kynurenine hydroxylase                          | ommochrome synthesis                          | RPRC001714-RA       |

Table I. Pigmentation-associated genes identified and functionally analyzed in this study.
Figure 4