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Genetics of a sex-linked recessive red eye color mutant of the tarnished plant bug, *Lygus lineolaris*

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ABSTRACT

An inbred colony of the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Miridae: Hemiptera), was observed to contain specimens with abnormal traits including red eyes, deformed antennae, and deformed legs. These specimens were isolated and back crossed to create stable phenotypic strains. The only successful strain established was a red eyed strain named Cardinal. The trait was more prevalent and stable in males, suggesting that it could be sex linked. To test the hypothesis that the trait was based on a recessive sex linked allele, classical genetic crosses were performed. The hypothesis was confirmed, and the eye color phenotype was measured and characterized using color analysis software. The trait is similar to other red eyed phenotypes described in this species, but is clearly based on a different mutation since it is sex linked rather than autosomal. The results of crossing experiments also suggest that inbreeding in this species results in substantial fitness cost to laboratory insects.

Keywords: Genetics; Inbreeding; Fitness; Pigmentation; Ommochromes; Antennae; Spineless

1. INTRODUCTION

Variations in the eye color of insects can be found in nature and selected by inbreeding or induced by mutagenesis. Naturally occurring and induced mutations have served as a foundation for progress in both general genetics and in genetic manipulation of insects. A white eye color mutation was found to be linked to sex in *Drosophila melanogaster*, defining the phenomenon of sex-linkage, and establishing methods in classical genetics [1]. Transplantation studies using *D. melanogaster* eye disks from multiple eye color strains were vital to the establishment of biochemical pathway genetics [2]. Insect eye colors were later found to be more complex than could be explained by the one-gene-one-enzyme principle, and were shown to be related to transporter pathways [3]. More recently, eye color mutations have been utilized extensively to verify manipulation systems involving genetic transformation [4,5], especially when the eye-specific promoter 3xP3 is utilized [6].

Eye color mutations have been described in numerous insects other than *D. melanogaster*. Within the order Diptera, eye color mutations have been described in fruit flies, family Tephritidae [7-9], in blow flies, family Calliphoridae [10-14] and house flies [15-17], in the tsetse fly [18-22], and in many mosquitoes [23-33]. Eye color mutations have also been described in the order Lepidoptera [34-40], in the order Coleoptera [41-43], and in the order Hymenoptera [44-46]. In the order Hemiptera, eye color mutations have been described in kissing bugs (family Reduviidae) [47-51], in the tarnished plant bug, the subject of this paper, family Miridae [52,53], and recently in the brown planthopper, family Delphacidae [54].

The rapid pace of progress in genomics and bioinformatics presents remarkable opportunities for discovery and analysis of insect genes. Efforts to capitalize on this progress will be enhanced by increased focus on basic biological function phenomena in insects that have not yet been developed as model organisms. Naturally occurring strains with genetically defined visible markers will once again serve as valuable resources to scientific progress. A red-eyed strain, established through inbreeding of *Lygus lineolaris*, was reported previously [53]. A similar strain, established from a wild male specimen collected in the field in Arkansas was reported more recently [52]. Through genetic analysis, the eye color allele in these naturally occurring strains were characterized as autosomal recessive.

As part of an effort to perform *in vivo* genetic manipulations on *L. lineolaris*, a laboratory strain was established in quarantine and inbred for 5+ years. This strain was examined regularly for unusual phenotypes that might indicate mutation. Phenotypes associated with abnormal appendages were observed but could not be reproduced and maintained in the laboratory through normal breeding. However, an eye color mutation was observed and isolated. To verify that this mutant strain was not the same as the ones previously described, reciprocal crosses were undertaken to test the hypothesis that the mutation was sex linked. Additionally, digital image analysis was utilized to characterize the color of the phenotype. To differentiate the phenotype from those previously described, the strain was called Cardinal, with the eye color mutation *cardinal*. Strains generated by inbreeding could be highly useful in forthcoming genomics projects using this species of pest insect and other arthropods.

2. MATERIALS AND METHODS

2.1. Insects

A wild-type colony was maintained without introgression for 5+ years in the Stoneville Research Quarantine Facility (SRQF) in Mississippi. Insect stocks were kept in an environmental chamber (Percival Scientific, Inc. Perry, IA) set for 16:8 (L:D) h lighting regime with 25˚C daytime and 19˚C nighttime temperature and 55%RH. Insects were provided a standardized diet and oviposition system based on NI diet and 4% gelcarin oviposition substrate [55].

2.2. Classical Genetics

For first generation matings small groups of virgin insects, two males and four females, were housed in isolation. Second generation insects were grouped in sets of two males and seven females. Insects were sexed in the 5th instar [52]. Eggs were collected daily. Mature eggs were separated from oviposition substrate and kept on moist filter paper for observation until hatch, then transferred to 100 mm by 15 mm Petri dishes for rearing to the adult stage. Nymphs were provisioned with fresh red clover leaves as dietary supplement and refugia.

2.3. Imaging

Images of living specimens were collected using a stereoscopic zoom microscope (Nikon SMZ1500) and Nikon digital camera (DMX 1200). Images used to analyze the eye color were cropped to include only eyes and converted to .jpg files then analyzed using RGB software [56]. Each image was analyzed three times using different portions of the eye image for analysis. The means of these samples were then used to generate color match indicators for illustrations.

2.4. Statistical Analyses

Egg production data were analyzed using the mixed procedure SAS Enterprise Guide v. 4.2 (SAS 2006). Phenotype and sex ratio data were analyzed for goodness of fit to Mendelian ratios using the Chi-Square test [57].

3. RESULTS

After inbreeding a culture of *L. lineolaris* without introgression for overlapping generations of roughly 60 days for four years (approximately 24 generations), a red eyed individual was identified in a colony cage. For the next two years backcrossing and inspection of the parent colony for additional red eyed stock eventually produced a homozygous strain of red eyed specimens (**Figure 1**). When the first red-eyed individuals were identified they were invariably male, and were paired with wild type females from the inbred source colony. When back crosses of the offspring were obtained, male red eyed individuals were found but red-eyed females were rare. Thus the possibility that the eye color was a sex linked phenotype was recognized early on. It took several generations to establish a homozygous colony of red-eyed specimens (Cardinal), and the overall fitness of the strain seemed low, compared to the wild type (empirical observation). As Cardinal strain was becoming established, additional rare phenotypes began to appear regularly in both the wild and Cardinal strains. These phenotypes appeared as shortened and deformed appendages, initially short antennae (**Figure 2**). These individuals were also backcrossed and crossed with one another, but the phenotype was never reliably reproduced in offspring. The most common deformity other than short antennae found in the inbred colonies was truncation of legs (**Figure 3**). These deformities sometimes coincided with the cardinal mutation, but also appeared in wild types with no apparent linkage. Because the phenotypes could not be established in permanent culture, no genetic data could be collected.

To test the hypothesis that fecundity was lower in the Cardinal strain, eggs were collected from small pools of individuals of wild type, Cardinal, and reciprocal crosses of the two strains. Egg production is shown in **Table 1**. While the differences in egg production were not significant in the first five or ten days of the test, by day 15, and continuing on day 20, the cumulative egg production data met the threshold ($P < 0.05$) to signify statistically significant difference (**Table 1**). Differences of least squares means on day 20 showed no significance between homozygous Cardinal (Ca × Ca) egg production and homozygous wild type (W × W) egg production ($P = 0.262$, $t = -1.24$, $df = 6$), and also no significant differ-

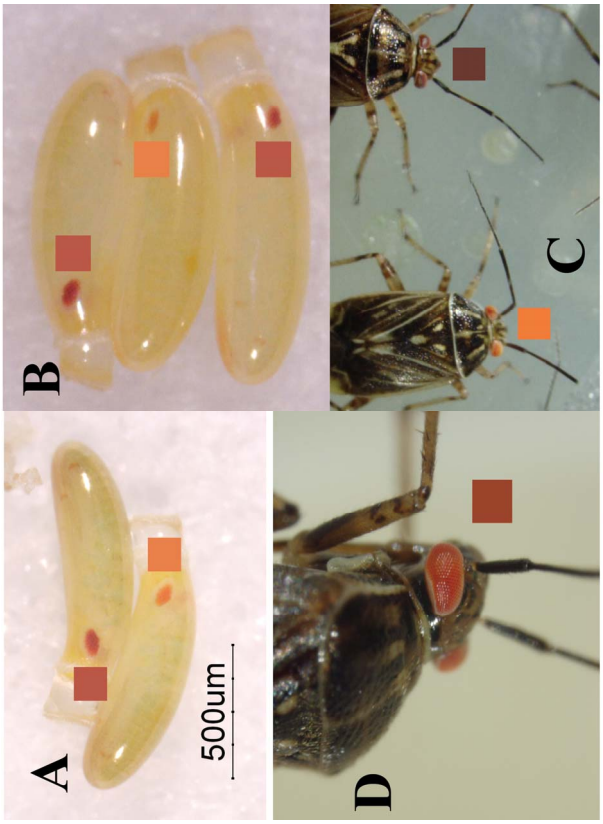

Figure 1. Eye colors of inbred strains of *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) with square color match indicator boxes. (A) Eggs produced by homozygous insects: normal eye color above, cardinal eye color below; (B) Eggs produced by crossing phenotypes: upper, normal eye color; middle, cardinal eye color from crimson female parent; lower, heterozygous (normal) eye color from crimson female parent; (C) Young adult eye color comparison: left, cardinal; right, normal (wild type); (D) Eye color of an older male (30+ days post adult eclosion/ecdysis).

ence between Ca x Ca and wild type male crossed with Cardinal female (W × Ca) (P = 0.291, t = 1.16, df = 6) nor between the W × W and W × Ca (P = 0.054, t = −2.40, df = 6). Additionally, the egg production of reciprocal crosses (W × Ca/Ca × W) did not differ from one another (P = 0.099, t = −1.95, df = 6). However, the Ca x W cross produced significantly lower quantities of eggs when compared to homozygous crosses W × W (P = 0.0048, t = −4.35, df = 6) and Ca × Ca (P = 0.021, t = 3.11, df = 6).

To test the hypothesis that the phenotype was sex linked, crosses of the Cardinal and wild type strains were evaluated, and yielded the expected ratios of offspring in the first generation for a recessive sex-linked mutation (**Table 2A**). All offspring of a male Ca parent were wild type, while all male offspring from a female Ca parent were red eyed and all female offspring were wild type. Second generation crosses of heterozygous females from both first generation crosses also yielded expected ratios

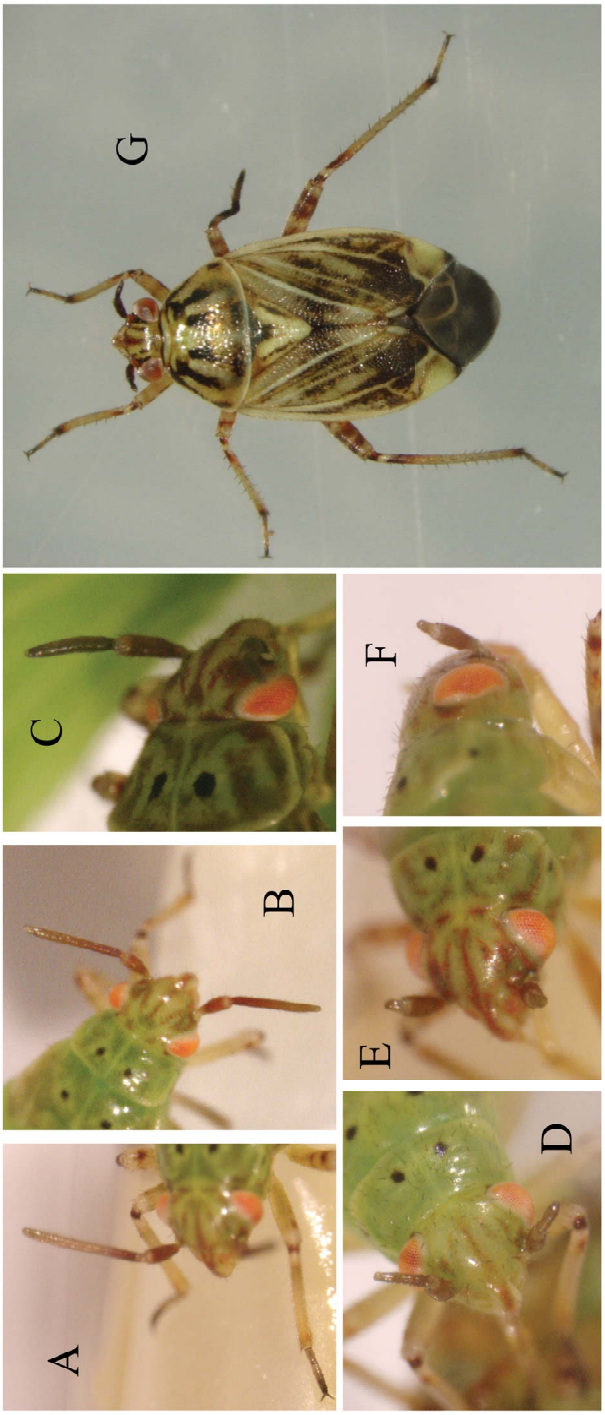

Figure 2. Inbred specimens of Lygus lineolaris that display deformed antennae. (A)-(F) nymphs; (G) adult. Note that the right middle leg of this specimen is also defective, tibia is shortened and tarsal segments are fused.

of offspring (**Table 2B**). The crosses are illustrated diagrammatically in **Figure 4**.

Overall survival to the imaginal stage from fertile em bryos was only evaluated in the F_1 mating pools: from the male Ca parent 55.6% of wild type offspring survived

Table 1. Mean cumulative numbers of eggs (±SE) produced per female in crosses of inbred wild-type (W) and inbred cardinal-eyed (Ca) specimens of *Lygus lineolaris*.

Cross	Day							
♂ × ♀	5	n[a]	10	n	15	n	20	n
W × W	13.08 ± 2.39	12	25.58 ± 2.10	12	39.25 ± 2.29	12	51.14 ± 4.29	10
Ca × Ca	12.33 ± 2.51	12	20.42 ± 2.92	12	31.08 ± 3.29	12	41.25 ± 3.44	12
W × Ca	7.33 ± 4.51	12	18.42 ± 8.42	12	24.75 ± 10.00	12	32.00 ± 11.40	11
Ca × W	5.33 ± 1.92	12	8.00 ± 2.74	12	12.58 ± 2.43	12	16.42 ± 1.06	11
F	3.17		4.23		7.07		6.83	
$P > F$	0.11		0.06		0.02		0.02	

[a]The degrees of freedom for each comparison was 3.39.

Table 2. First and second generation crosses of Cardinal eye color strain to parental wild type strain. (A) First generation heterozygous female parents are either combined or identified by the sex of the Cardinal-eyed parent. Second generation combined analysis included with pairwise comparisons of phenotype and male/female ratio. Critical value for all chi-square tests is 3.84, df = 1; (B) Second generation crosses, combined data (above) and data analyzed by parent carrying cardinal allele. Critical value for phenotype data is 7.82, df = 3.

A.				adult phenotype						adult survival					
Cross	egg phenotype		χ^2	wild type		χ^2	cardinal		χ^2	Totals		χ^2	Totals		χ^2
♂ × ♀	wild type	cardinal		♂	♀		♂	♀		wild type	cardinal		♂	♀	
W × W	580	0	-	202	166	0.880	0	0	-	368	0	-	202	166	0.880
Ca × Ca	0	537	-	0	0	-	248	231	0.151	0	479	-	248	231	0.151
W × Ca	265	228	0.694	0	143	-	136	0	-	143	136	0.044	136	143	0.044
Ca × W	337	0	-	117	141	0.558	0	0	-	258	0	-	117	141	0.558
Ca × F_1	438	397	2.013	103	134	4.055[a]	131	125	0.141	237	256	0.732	234	259	1.268

B.				adult phenotype					adult survival					
Cross	egg phenotype		χ^2	wild type		cardinal		χ^2	Totals		χ^2	Totals		χ^2
♂ × ♀	wild type	cardinal		♂	♀	♂	♀	(df = 3)	wild type	cardinal		♂	♀	
Ca × F_1	438	397	2.013	103	134	131	125	4.777	237	256	0.732	234	259	1.268
Ca × $F_{1♂}$	245	208	3.022	59	78	75	58	4.874	137	133	0.059	134	136	0.015
Ca × $F_{1♀}$	193	189	0.041	44	56	56	67	4.749	100	123	2.372	100	123	2.372

[a]Exceeds critical value for chi-square tests, 3.84, df = 1.

and 64.9% of cardinal offspring survived; from the female Ca parent 69.5% of wild type offspring survived and 70.5% of cardinal offspring survived. No significant difference was detected ($P > 0.05$, student t-test, df = 2).

Eye colors of the wild-type embryonic insects corresponded to RGB colors at R 193 ± 8, G 88 ± 7, B 70 ± 10 while the mutant embryonic eye colors corresponded to RGB colors R 240 ± 2, G 130 ± 7, B 68 ± 10. These colors persisted as developing embryos, nymphs, and through early adult stage. As the adults darken with age, particularly the males, the eyes darken to a more crimson color corresponding to R 161 ± 7, G 70 ± 18, B 44 ± 20. The fully mature wild-type adult eye corresponds to R 110 ± 7, G 58 ± 8, B 48 ± 6 (**Figure 1**).

4. DISCUSSION

Standardized laboratory rearing of *L. lineolaris* is primarily for the purpose of bioassays. The cultures of insects are expected to represent field performance of naturally occurring populations. Thus, colonies are frequently intermixed with new field collected insects in order to maintain colony health and heterozygosity [58]. The laboratory colony in the SRQF differed in that no

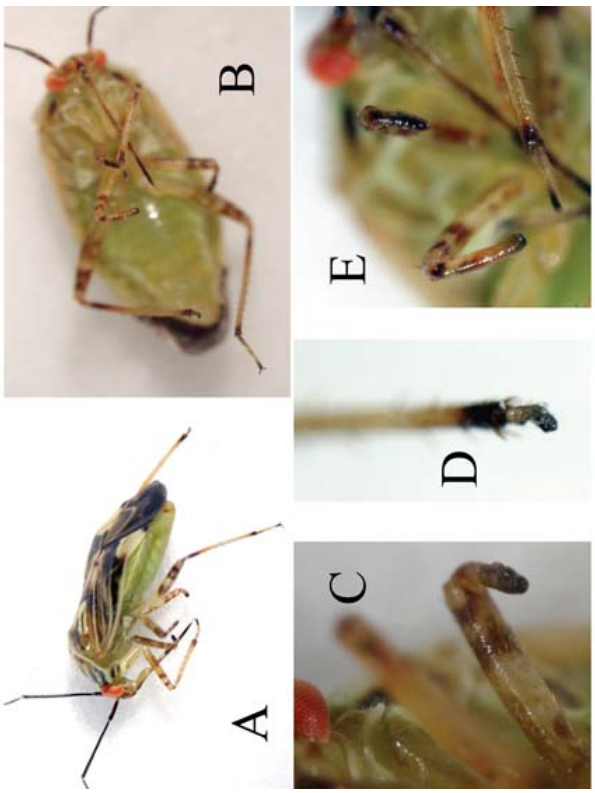

Figure 3. Individual specimen from Cardinal strain with severe leg deformities. Four of six legs have shortened and deformed termini. Tarsal segments appear to be fused. (A) Right lateral view; (B) Ventral view; (C) Left middle leg terminus; (D) Right hind leg; (E) Right foreleg and middle leg termini.

introgression by field collected insects was allowed. This culture strategy was specifically intended to limit heterozygosity and provide more inbreeding to support genetic analysis and genetic manipulation. Under laboratory inbreeding conditions, spontaneous mutations often are revealed. Novel eye color phenotypes are not uncommon in colonies of insects. Those arising in colonies of *D. melanogaster* kept by Thomas Morgan and his students formed a foundation for modern genetics.

The truncated appendages observed repeatedly in the inbred colony bear resemblance to defects described in *D. melanogaster* and *Tribolium castaneum* related to the transcription factor *spineless* [59-61]. Further careful breeding of similar specimens will be required to determine whether these phenotypes are related. Unfortunately, the specimens we found were delicate and generally infertile, and were discarded after multiple attempts to establish a stable strain failed.

Naturally occurring orange or red eye color phenotypes of Hemiptera have been described [47-50,52-54]. Eye pigments in two species of Hemiptera in the family Reduviidae differ from one another, those from *Triatoma infestans* Klug being composed primarily of xanthommatin [47], while the eye pigments of *Rhodnius prolixus*

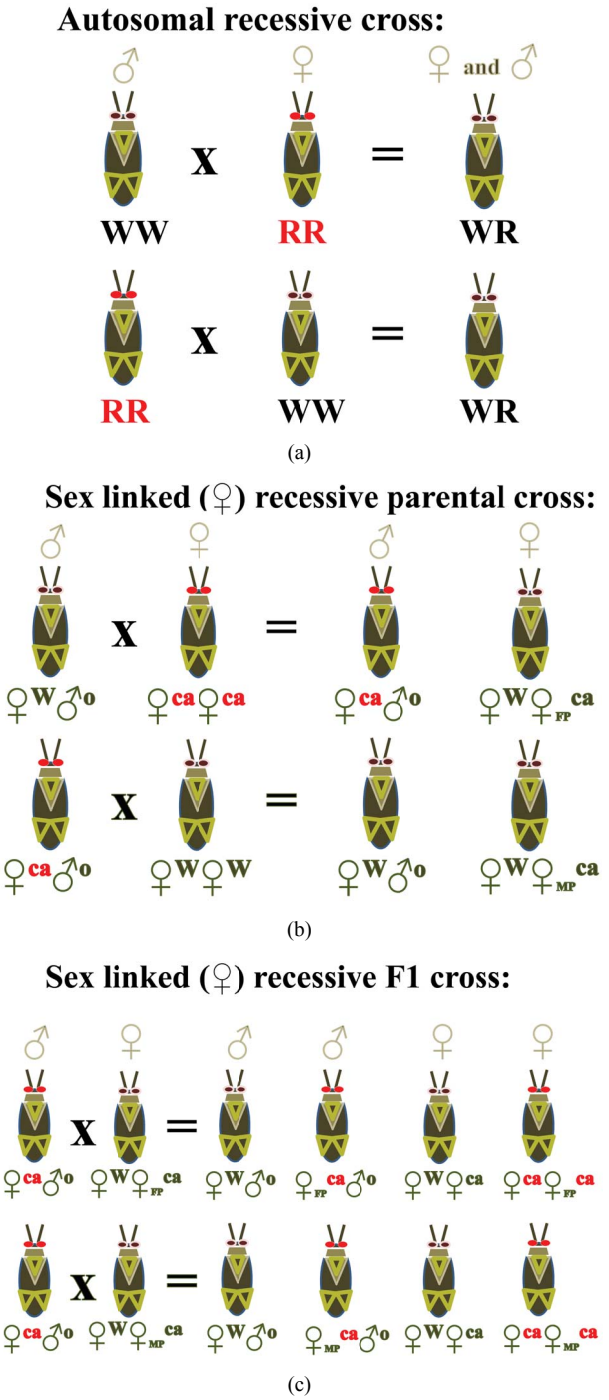

Figure 4. Diagrams showing expected results from crossing wild type insects with insects carrying an autosomal recessive red eye trait (R) compared with results from crossing wild type insects (WT) with insects carrying a sex linked recessive red eye trait such as cardinal (ca). MP = allele from male parent, FP = allele is from the female parent.

are composed primarily of ommins [62]. The only enzyme identified from *L. lineolaris* from the ommochrome biosynthetic pathway is tryptophan oxygenase (TO) (http://www.ncbi.nlm.nih.gov/nuccore/307634529 and

http://www.ncbi.nlm.nih.gov/protein/307634530, submitted 10 August 2010, accessed 31 July 2012). This enzyme catalyzes the initial reaction in the conversion of tryptophan to ommochromes, the pigments in insect eyes. The *white* gene used to define sex linkage by Morgan (1910) was shown to be a membrane pigment transporter gene rather than a pigment synthesis enzyme gene (Sullivan and Sullivan 1975), and this may also be the case in *L. lineolaris*. The genetic identity of TO has been characterized in *D. melanogaster* [63], and the red flour beetle *Tribolium castaneum* [64], and putative homologues with a high similarity at the nucleotide level [65] can be identified in the human body louse *Pediculus humanus corporis* (XM_002423485.1, accessed from NCBI 16 July 2012) and the mountain pine beetle *Dendroctonus ponderosae* (BT128347.1, accessed from NCBI 16 July 2012) [66]. While TO may be mutated in either the autosomal or sex-linked phenotypes of red eyed *L. lineolaris*, any number of other biosynthesis or transport genes may also be responsible.

Both naturally occurring eye color strains of *L. lineolaris* previously identified were characterized as autosomal recessive alleles. The wild collected eye color variant [52] was reported to have little effect on behavior or physiology, although because of the low field prevalence of the phenotype it was implied that there could be a fitness disadvantage, possibly associated with mating disadvantage based on decreased visual ability. The red-eyed "R" strain was kept in culture for >5 years, but has since been discarded (G. Snodgrass, personal communication). The eye color mutation identified through inbreeding [53] was not tested for physiological characteristics, but was observed to display no obvious behavioral or developmental differences from wild type. The strain reported here and the progenitor wild type strain both exhibited apparent loss of fitness. The 20 day cumulative egg production reported for the wild caught red strain was over 120 eggs/female, while the specimens reported here produced less than half that in every cross (**Table 1**). The loss of fecundity is most distinct in the fecundity of the Ca × W cross. The reason for this loss of fecundity is not known. Interestingly, the total number of male vs. female wild type offspring produced by the Ca × F1 crosses exceeded the critical value for the expected m/f ratio (**Table 2A**), while the overall phenotype distribution fit the expected results (**Table 2B**). Inbreeding depression is an accepted phenomenon, relevant to evolution, conservation, and agriculture. Intentional inbreeding is also expected to play a vital and dominant role in future molecular genetics and functional genomic research [67].

If genetic manipulation technology improves to a point at which sex-specific applications such as the sterile insect technique (SIT) can be applied to insects in the order Hemiptera, identification of sex-specific genes and gene regulation will become critical to project success. Visible markers that can be induced by inbreeding or other methods of mutagenesis will facilitate progress towards these applications, and towards a better understanding of the genetics of these important pest insects.

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