University of Huddersfield Repository

Reeves, R. Guy, Bryk, Jarek, Altrock, Philipp M., Denton, Jai A. and Reed, Floyd A.

First Steps towards Underdominant Genetic Transformation of Insect Populations

Original Citation

Reeves, R. Guy, Bryk, Jarek, Altrock, Philipp M., Denton, Jai A. and Reed, Floyd A. (2014) First Steps towards Underdominant Genetic Transformation of Insect Populations. PLoS ONE, 9 (5). e97557. ISSN 1932-6203

This version is available at http://eprints.hud.ac.uk/id/eprint/25624/

The University Repository is a digital collection of the research output of the University, available on Open Access. Copyright and Moral Rights for the items on this site are retained by the individual author and/or other copyright owners. Users may access full items free of charge; copies of full text items generally can be reproduced, displayed or performed and given to third parties in any format or medium for personal research or study, educational or not-for-profit purposes without prior permission or charge, provided:

- The authors, title and full bibliographic details is credited in any copy;
- A hyperlink and/or URL is included for the original metadata page; and
- The content is not changed in any way.

For more information, including our policy and submission procedure, please contact the Repository Team at: E.mailbox@hud.ac.uk.

http://eprints.hud.ac.uk/
First Steps towards Underdominant Genetic Transformation of Insect Populations

R. Guy Reeves1*, Jarosław Bryk1, Philipp M. Altrock2, Jai A. Denton1, Floyd A. Reed1

1 Department of Evolutionary Genetics, Max Planck Institute for Evolutionary Biology, Plön, Germany, 2 Department of Evolutionary Theory, Max Planck Institute for Evolutionary Biology, Plön, Germany

Abstract

The idea of introducing genetic modifications into wild populations of insects to stop them from spreading diseases is more than 40 years old. Synthetic disease refractory genes have been successfully generated for mosquito vectors of dengue fever and human malaria. Equally important is the development of population transformation systems to drive and maintain disease refractory genes at high frequency in populations. We demonstrate an underdominant population transformation system in Drosophila melanogaster that has the property of being both spatially self-limiting and reversible to the original genetic state. Both population transformation and its reversal can be largely achieved within as few as 5 generations. The described genetic construct (Ud) is composed of two genes; (1) a UAS-Rpl14 dsRNA targeting RNAi to a haploinsufficient gene Rpl14 and (2) an RNAi insensitive Rpl14 rescue. In this proof-of-principle system the UAS-Rpl14 dsRNA knock-down gene is placed under the control of an Actin5c-GAL4 driver located on a different chromosome to the (Ud) insert. This configuration would not be effective in wild populations without incorporating the Actin5c-GAL4 driver as part of the (Ud) construct (or replacing the UAS promoter with an appropriate direct promoter). It is however anticipated that the approach that underlies this underdominant system could potentially be applied to a number of species.

Introduction

Genetic underdominance at a given locus occurs when heterozygotes have a lower fitness than both homozygotes; i.e. heterozygote disadvantage [1,2]. The mechanism of population transformation can be illustrated for an underdominant transgenic construct (Ud). If it is assumed that both wildtype (+/+ ) and transgenic homozygotes (Ud/Ud ) have equal fitness (though this is not a necessary requirement), and if the frequency of the Ud allele is raised above a 0.5 threshold frequency by releases of Ud/Ud heterozygotes into a target wildtype population, then Ud alleles will likely eliminate wildtype alleles at this locus over the course of subsequent generations. The elimination of wildtype alleles can occur even after releases have ceased [1,3–5]. This mechanism is a consequence of the Hardy-Weinberg principle. The Hardy-Weinberg principle states that the rarer wildtype alleles (+) spend proportionally more time as low fitness heterozygotes than the Ud allele [6]. This population transformation process is inherently reversible, as wildtype homozygotes could also be released to traverse the population frequency threshold in the opposite direction. Consequently, underdominant population transformation can be thought of as an evolutionary bi-stable switch. The switch works such that exceeding a threshold frequency dictates which allele will ultimately be fixed. Crucially, the transgenic homozygote can be less fit than the wildtype homozygotes and still attain stable fixation. This results in the three well-known properties of underdominant population transformation [3–5,7–9]: (1) geographically self-limiting to specific populations, (2) reversibility to original wild state, and (3) linkage to deleterious transgenes does not prevent population transformation. It is important to note that the driving effect of Ud will be centered on its insertion site with the remainder of the genome being free to recombine with wildtype chromosomes.

The implementation of the Ud system described here relies on a knock-down and rescue system in a transgenic construct. Our transgenic construct targets an endogenous haploinsufficient gene (with expression control of the knock-down gene divided between two loci using the GAL4/UBAS system, [10,11]). Haploinsufficient genes are ones where null (amorphic) or hypomorphic mutations cause a dominant phenotype. A well-studied class of haploinsufficient genes are the D. melanogaster Minute loci (reviewed in [12]). Our approach relies on dominant RNAi-based suppression of an endogenous (present in the wildtype genome) haploinsufficient gene. The RNAi mediated knock-down of the endogenous gene is rescued by an RNAi-insensitive copy of the gene also in the same
phenotype with delayed development, slender scutellar bristles and individuals, could be used to push disease refractory genes into insects at a sufficiently high frequency through mass release of humans and mice (see references in [12,14]).

The endogenous haploinsufficient gene targeted in the construct described here is RpL14 (located on chromosome arm 3L with a cytogenetic location of 66D8), which is a cytoplasmic ribosomal protein (CRP). Heterozygous amorph or hypomorph mutations in RpL14 result in a classic strong Minute phenotype with delayed development, slender scutellar bristles and reduced female fertility [13]. It is noteworthy that haploinsufficient mutations in the 88 CRP genes of D. melanogaster very likely correspond to 64 of the 65 known Minute loci [12]. Haploinsufficiency of CRP genes has been reported in a wide range of organisms including yeast, Arabidopsis, Drosophila, zebrafish, humans and mice (see references in [12,14]).

Herein we report a transgenic construct {Ud}, a simple modification that, when introduced into target populations of insects at a sufficiently high frequency through mass release of individuals, could be used to push disease refractory genes into wild populations.

Materials and Methods

Development of UAS-dsRNAi.RpL14 Knock-down of RpL14[14]

The RpL14 gene (CG6253, FBgn0017579) was selected as a representative CRP gene, which had previously been described as exhibiting a strong to extreme Minute phenotype [13]. To identify an approximately 70 bp region of RpL14 to target by RNAi knock-down we sought to fulfill the following four criteria in the wildtype exon sequence: (1) No predicted off-target effects (default setting with 'Off-Target Size' set to 16 bp, http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl), (2) No regions with a high number of non-degenerate codons ATG or TGG, (3) target must be present in all alternative splicing variants, and (4) the region exhibits a high degree of structural accessibility (according to default settings of S-fold, http://sfold.wadsworth.org/cgi-bin/srna.pl). When these criteria were applied it was not possible to target a single contiguous region. Hence two non-contiguous regions were selected (called block A and B). Both regions happened to be on exon 2. The sequence of RpL14 dsRNA is shown in Figure S2 in File S1. An inverted repeat of the targeting sequence was cloned into a plasmid in the HindIII site immediately upstream of the UAS promoter in the pUASattB plasmid already containing the UAS-RpL14 dsRNA sequence (see above). Figure 1. It may be noteworthy that two intronic snoRNAs were also cloned as part of the fragment, though only exonic sequences are targeted by the dsRNAi.RpL14 gene.

The 14 synonymous mutations that conferred insensitivity of RpL14[14] to RNAi targeting were introduced into exon 2 by synthesizing a new sequence (DNA synthesis done by DNA2.0, Menlo Park, USA). This was then ligated into a plasmid in the place of the corresponding wildtype sequence, using standard cloning techniques. As far as possible, the introduced mutations preserved the same balance between D. melanogaster preferred and unpreferred codons. The insensitivity of RpL14[14] to RNAi targeting and its effectiveness as a phenotypic rescue gene was demonstrated by its ability to rescue the otherwise 100% lethality of UAS-RpL14 dsRNA in the presence of Act5C-GAL4 expression (see Figure S4b in File S1).

Generation of outbred transgenic stocks

Germline transformants were generated by inserting the {Ud} plasmid (Figure 1) into the 86Fb (3R:7634329, [17]) landing site (injections done by BestGene, Chino Hills, USA). Heterozygous transformants y{1} w^{sp}, M(3x-P3-RFP, attR, w^{sp}, Rpl14[14], UAS-RpL14 dsRNA, attL), 67Bf/+ were outcrossed for >3 generations to a mixed assortment of the following wild derived stocks, the wildtype lines are Bloomington Drosophila Stock Center No. 3845, CO 3 (NY, USA); Bloomington No. 3883, Wild 5A (GA, USA); Aquadro lab (Cornell University), B96 (Beijing, China).

Figure 1. Configuration of the proof of principle underdominant transgenic construct {Ud}. The first gene of the construct encodes a dsRNA RNAi knock-down (orange) under the control of a UAS promoter 10 (magenta). The dsRNA targets RNAi to 72 bp of exon 2 of the endogenous wildtype gene RpL14[14]. The second gene (green) of the construct is a rescue gene RpL14[14] that is insensitive to the RNAi knock-down (see Figure S1 in File S1 for strategy overview). RpL14[14] is a complete RpL14 gene, including all its original regulatory regions, into which 14 synonymous mutations (14*) have been strategically introduced in exon 2 (Figure S3 in File S1). Expression of the RNAi knock-down is under the control of an Act5c-GAL4 driver at a remote locus.

doi:10.1371/journal.pone.0097557.g001

Development of rescue RpL14[14]

The RpL14 region was amplified with Phusion Taq (Finnzyme) using DNA prepared from the genome reference stock (Bloomington Drosophila Stock Center No. 2057: y{1}, G{22D1} G{22F1} or{1} CG3296{2R2} lac{1} y{12}; I{2}G{2} M{2}G{2} D{3} R{2} F{2}). This was done using the following primers NotI-5' TATGCGGCCGCctgattacttcgccact and EcoRI-5' TATGAATTCaaggcataagagcttt-. This resulted in amplification of 3L: 8593592..8596494. To help ensure that all unidentified regulatory regions of RpL14 were incorporated, fragments of the flanking genes were also included in the PCR product. The endogenous RpL14 fragment was cloned into the HindIII site immediately upstream of the UAS promoter in the pUASattB plasmids already containing the UAS-RpL14 dsRNA sequence (see above). Figure 1. It may be noteworthy that two intronic snoRNAs were also cloned as part of the fragment, though only exonic sequences are targeted by the dsRNAi.RpL14 gene.
and 9 stocks acquired from the wild in 2009 in Kiel and Plön, Germany. The crossing scheme outlined in Figure S5 in File S1 details subsequent steps in the generation of the following two stocks that were used in varying frequencies to initiate all population experiments: {Act5C-GAL4}/CyO; {UD}86/{UD}86 and {Act5C-GAL4}/CyO; +/+ . As the genotypes differ only on the third chromosomes, only the third chromosomes genotype is generally used in the main text.

Note that while all chromosomes were outcrossed for >3 generations, the maintenance of the second chromosome balancer CyO in the experiments keeps the original outbred second chromosomes in a permanently heterozygous state preventing the large fitness cost (≥90%, [18]) associated with chromosomal isoenicity. This will also have the effect of preventing recombination during the population experiments on the second chromosome, which will not be the case for the first and third chromosomes.

The stocks used to establish the experimental outbred populations were sequenced to detect any variation in the RNAi targeted region (3L:8594414-8594515). Using PCR primers 5′-TGGTCACCAACACAAAGC and 5′-GCCATGAAAGGGAGGAGTACA, sequenced stocks included Bloomington stock 3848, CO 3 (NY, USA); Bloomington stock 3805, Wild 5X (GA, USA); Aquadro lab, BJ96 (Beijing, China). Seven of the 9 stocks acquired from the wild in 2009 in Kiel and Plön (Germany) were also sequenced (2 stocks had died prior to sequencing). No polymorphism was detected. Sequencing also detected no polymorphism in 5 stocks resulting from the endpoints of the experiments shown in Figure 2. These stocks had been maintained as bottle populations for more than a year after the end of the experiment. DNA was prepared from pools of 10 individuals for each of the 5 stocks, PCR products were generated and cloned. A total of 20 Rpl14 66D8 derived clones were sequenced and found to be invariant. The complete lack of polymorphism detected is consistent with the levels of variation observed in this region in large scale sequencing projects. For example based on 156 individuals with complete sequence data for this region there is just one polymorphic site (sampled in just two individuals [19]).

Epi-fluorescent genotyping of flies

Genotyping of flies was done by surveying for the RFP fluorescence of the 3xP3-RFP gene at the {UD}86 landing site. Scoring of fluorescence was done on a Leica MZ10 F epi-fluorescent microscope with a Leica EL 6000 light source in a fluorescence of the gene at the {UD}86 landing site. Fluorescence microscopy, and then allowed to lay eggs in a new bottle for 3 days before being cleared. Following 12 additional days of larval development, up to (approximately) 100 G2 offspring were collected scored for sex and RFP using epifluorescence microscopy, and then allowed to lay eggs in a new bottle for 3 days before being cleared. Following 12 additional days of larval development, up to (approximately) 100 G2 offspring were collected scored for sex and RFP and introduced into a bottle for three days. This sequence was repeated for all subsequent generations. The G2 generation is the first in which heterozygotes are present (Figure 2a) and consequently it is the first generation in which an underdominant effect could be detected. This is why generation 2 is the first generation shown in Figure 2a.

An egg laying time of 3 days and generation spacing of 15 days, from the time of initial addition of flies to each bottle, was chosen to maximize population sizes and minimize possible effects of developmental delay, i.e. the balance between early eclosing genotypes dying in the food and thus underscored and later eclosing genotypes being underscored. Using the curve of development time, Figure S4a in File S1, the observation that eggs were consistently observed to have been laid on all three days, and assuming 100% homozygous genotype survival, this would only result in a relative loss of 7% of the expected heterozygous genotypes. This is not sufficient to explain the 78% fitness reduction estimated for heterozygotes, which as discussed in the main text is likely to result from a combination of pleiotropic effects of reduced CRP expression of in some tissues (see also Figure S1 in File S1).

Population experiments

The experiment to measure allele frequency change over multiple generations was initiated with 20 mated females from the above two outbred homozygous stocks, at either 20%, 50% or 80% initial {Act5C-GAL4}/CyO; {UD}86/{UD}86 frequencies (UD]86 homozygotes). The remainder of individuals were {Act5C-GAL4}/CyO; +/+ (wildtypes). These already mated females were allowed to lay eggs in vials for three days and then cleared. These homozygous female parental flies were termed the G0 generation. The purpose of this generation was to minimize the possibility that differences in the condition or maturity between stocks did not strongly bias starting frequencies. The next generation, G1 was genotypically identical to generation G0, but larval development conditions are better controlled. Up to 100 G1 adults from a vial were collected, scored for sex and RFP using epifluorescence microscopy, and then allowed to lay eggs in a new bottle for 3 days before being cleared. Following 12 additional days of larval development, up to (approximately) 100 G2 offspring were collected scored for sex and RFP and introduced into a bottle for three days. This sequence was repeated for all subsequent generations. The G2 generation is the first in which heterozygotes are present (Figure 2a) and consequently it is the first generation in which an underdominant effect could be detected. This is why generation 2 is the first generation shown in Figure 2a.

An egg laying time of 3 days and generation spacing of 15 days, from the time of initial addition of flies to each bottle, was chosen to maximize population sizes and minimize possible effects of developmental delay, i.e. the balance between early eclosing genotypes dying in the food and thus underscored and later eclosing genotypes being underscored. Using the curve of development time, Figure S4a in File S1, the observation that eggs were consistently observed to have been laid on all three days, and assuming 100% homozygous genotype survival, this would only result in a relative loss of 7% of the expected heterozygous genotypes. This is not sufficient to explain the 78% fitness reduction estimated for heterozygotes, which as discussed in the main text is likely to result from a combination of pleiotropic effects of reduced CRP expression of in some tissues (see also Figure S1 in File S1).

Population experiment statistical tests

The strategy of scoring a dominant RFP marker to infer the frequency of {UD}86 makes minimal assumptions regarding fitness. The frequency of {UD}86 was estimated as \( p = 1 - (R^n) / n \), where \( R \) is the fraction of non-RFP expressing adults and \( n \) is the total number scored (plotted in Figure 2a; data is given in Table S1 in File S1). A likelihood-ratio test, related to but having better properties than the standard \( \chi^2 \) test [20], was used to analyze the counts of RFP and non-RFP males and females between generations and search for maximum-likelihood parameter value estimates over a grid of transgenic homozygote and heterozygote fitness values (relative to a wildtype fitness set to one). This was done by minimizing the G-statistic of a likelihood-ratio G-test [21,22] for predicted changes in frequency between generations [cf. [23]] according to the fitness of each genotype and their corresponding frequency in the population in non-overlapping, random-mating Wright-Fisher generations [24]. According to standard deterministic Wright-Fisher model assumptions the expected allele frequency in the next generation \( p' \) is given by the allele frequency in the current generation \( p \), the homozygote and heterozygote fitness values \( w_{AA}/(1-Ud) \) and \( w_{Aa}/(1-Ud) \), and the average fitness in the population, \( \bar{w} \). Change in allele frequency then follows.
For a given set of fitness values this generates an expected value that the observed data can be compared to in a likelihood framework [25]. Assuming independence, both in allele frequency changes across replicates and between sets of generations, allows the sum of the log-likelihood ratios of individual frequency changes to be used to generate a joint/composite likelihood surface over the full data set (equivalent to multiplying probabilities using the independence rule). According to Wilks’ theorem, and the assumption that the errors are normally distributed according to the central limit theorem of many small effects, the test-statistic is expected to be asymptotically \( \chi^2 \) distributed [26], which was used to generate joint confidence intervals (plotted in Figure 2b). Profile likelihoods derived from the joint surface were used to generate the confidence intervals for individual parameter estimates (plotted as error bars in Figure 2c).

Confirming homozygosity of stocks

Homozygosity was confirmed by crossing large numbers of \( n > 30 \) tester males to virgin females (from a \( w^{[1118]/, \ Df(2;Y)G^{hs-hid}} \) virginizer stock) and determining whether all offspring were RFP fluorescent or all non-fluorescent. In addition the following primers were used on single fly DNA extractions 5’ gggccaaagtgtaaataactgg and 5’ aaaatgtccattactttggtgct to give a 136 bp PCR product (3R:7634237..7634372) identifying the presence of wildtype third chromosomes (+) and a theoretical product of \( >10 \) kb for \( \{Ud\}_{86} \). Presence of \( \{Ud\}_{86} \) could be

\[
p' = \left( \frac{w_{\{Ud\}}}{w_{\{Ud\}} + p^2} \right) p(1 - p) + \frac{w_{\{Ud\}}}{w_{\{Ud\}} + p^2} p(1 - p) = \frac{\bar{w}}{\bar{w}}
\]
confirmed by using 5’ actcttccccatgaggacct and 5’ aaggccacgctcttc- 
cage resulting in a 135 bp PCR product from the RFP gene.

Development time and non-mendelian distortion
In order to more precisely quantify development time and egg- 
to-adult departure from expected Mendelian ratios among the three 
genotypes, a stock was generated with GFP expression from the 86Fb cytological insert site as a proxy for wildtype. This GFP 
stock had the genotype w; y; FRT1; P[w+mC ˚] = Act5C-GAL4 25F01; 
M[3x-P5-GFP]86Fb and was generated from a modification of a plasmid [pGFP-loc-attB 12.gb.1] kindly given to us by Dr. Johannes 
Bischof (University of Zurich).

Vials were used in this experiment and crosses were set up 
according to the parental types indicated in Figure S4b in File S1. 
The parental flies were transferred to new vials each day, for a 
total of 10 days, and the resulting newly eclosing offspring were 
scored, for sex and RFP/GFP presence/absence, each day over 
the following 25 days. The data is given in Table S2 in File S1.

The heterozygous to homozygous parental back-crosses allowed 
the rate of erroneous genotype scoring to be estimated. Vials for 
each cross were given an alphanumeric identifier and offspring 
were scored “blind” each day without knowledge of the parental 
sex or 10 unsexed larvae using Trizol (Invitrogen) and DNase 
treated using a PureLink RNA Mini Kit (Invitrogen). RNA quality 
was assessed with a two-step quantitative reverse-transcription 
quarqPCR reactions. We tested these genes for stability of expression in 
across 6-log serial dilutions of cDNA template and identified 
three of these genes, Fbg0032882 (CG9320), Fbg0039259 
(CG11781) and FBgn0002021 (CG10655) as the least variable in 
the flies and used these as normalization genes for the calculation of the 
normalization factor. RpL14 mRNA levels are presented relative to the normalized geometric mean of the three normal- 
ization genes.

Data deposition
The sequence and {Ud} plasmid is available from addgene.org 
with the ID 53220 and name {Ud}RpL14.Dm.

Results
The {Ud} construct
This system achieves underdominance through a “knock-down/ 
rescue” system contained in the construct {Ud} (Figure 1). {Ud} 
employs dsRNAi knockdown (RpL14 dsRNA) of an endogenous 
CRP, RpL14, driven by the UAS promoter [10]. This knock-down 
gene encodes a dsRNA inverted repeat, targeting RNAi to 72 base 
pairs (bp) of the endogenous RpL14 wildtype exons (RpL14 4˚), no 
intronic bases are targeted by the dsRNA inverted repeat. The 
construct also contains a rescue (RpL14 4˚) that is a complete copy of the 
wildtype RpL14 gene (including its promoter and flanking 
regions, to maximize the likelihood that rescue is complete) where 14 
synonymous mutations had been introduced within the 72 bp 
region targeted for RNAi by RpL14 dsRNA (Figures S2 and S3). 
The number and position of synonymous changes ensures that all 
~21 bp siRNA fragments potentially produced by Dicer proteins 
for RNAi targeting incorporated a minimum of 3 mismatches 
relative to the rescue gene RpL14 4˚, intended to render it 
substantially insensitive to RNAi targeting.

In this proof-of-principle system of underdominance, the 
GALA/UAS system [10] has been used to control expression of 
RpL14 dsRNA. This was done to permit flexibility in testing the 
promoters used for driving the RNAi knock-down expression and has 
the added benefit that the system would quickly be decoupled 
in the offspring of any accidental escaped flies. However, as 
the GAL4 driver is not part of the {Ud} construct in this example 
implementation the system is split across two loci and therefore 
RpL14 dsRNA is only expressed in a GAL4 background.

{Ud} Genomic integration & stain development
{Ud} was integrated at an RFP marked attP/C31 landing site on 
chromosome three ([17]; cytogenetic location 86Fb), resulting in 
the genotype M(3x-P5-RFP); {attB, w+mC ˚}; RpL14 4˚, UAS- 
RpL14 dsRNA, attL}86Fb, hereafter referred to as {Ud}86. To drive strong expression of the UAS-RNAi knock-down in at least 
some tissues, which can potentially experience haploinsufficiency, 
a constitutive second chromosome Act5c-GAL4 driver was selected [15] P[w+mC ˚] = Act5C-GAL4 25F01.

Phenotype of {Ud}86
Given the strong fitness reduction in heterozygotes and the 
pleiotropic impact of CRP hypomorphs [12,13], we examined life 
history and morphological traits that could correlate with this 
genotype. Two red-eyed phenotype stocks were generated for 
these experiments: {Act5C-GALA} C0; {Ud}86/{Ud}86 and; 
{Act5C-GALA} C0; (GFP)86/(GFP)86. We unambiguously
scoring all three genotypes in each generation using the fluorescence of the 3x-P3-RFP marker, which is part of the landing site used in {Ud}86, and the fluorescence of GFP inserted into the same position to mark the alternative chromosome as a proxy for wildtype. We observed no morphological abnormalities in either transgenic genotype. Interestingly, heterozygotes do not exhibit the short and thin scutellar bristles that are a characteristic feature of the Minute phenotype and most D. melanogaster CRP mutations. Furthermore, no difference in dry weight was observed between adults of the three genotypes (males, \( F = 0.55; P = 0.591 \); females, \( F = 1.34; P = 0.298 \), Table S3 in File S1). However, in common with Minute phenotypes, heterozygotes exhibit a development time prolonged by approximately 20 hours (Figure S4a in File S1, \( T < 1 \times 10^{-5} \)), while homozygotes \{Ud\}86/{Ud}86 exhibit no significant differences from wildtype homozygotes (Figure S4a and Table S2 in File S1). Also, the relative egg–to–adult viability of the heterozygous genotype was 20% to 50% lower than in homozygotes (Figure S4 and Table S2 in File S1).

Population transformation using \( \{Ud\} \)

To more directly test genotype fitness over the entire life-cycle, without possible fitness bias effects due to expression of a fluorescent marker in the wildtype stock, an unmarked red-eyed stock containing a balanced GAL4 second chromosome was generated for subsequent experiments (Figure S5 in File S1): \{Act5C-GAL4\}/CyO: +/+; \{Ud\}86/\{Ud\}86. Note that if there is a fitness effect of RFP expression on our \{Ud\}86 homozygote fitness estimate relative to wildtype is likely to be conservative. This wildtype-proxy stock is genotypically equivalent to our functional \{Ud\}86 stock \{Act5C-GAL4\}/CyO: \{Ud\}86/\{Ud\}86 in every way, including the balanced GAL4 driver on the second chromosome, except for the absence of the \{Ud\}86 insert. To ensure a robust test of underdominance, both stocks were initially out-crossed for 3 generations to a mixed stock composed of globally derived lines. This reduced the probability of large fitness losses in “wildtype” individuals due to isogenisity [18], which might artificially inflate the relative fitness of the \{Ud\}/\{Ud\} homozygote (note that the outbred second chromosomes are maintained in a permanently balanced state preventing recombination on this chromosome, see materials and methods for additional discussion).

To look for the frequency dependent fixation of alleles that is diagnostic of underdominance, replicated population bottles over a range of frequencies of \{Ud\}86 and + were initiated (Figure 2a and Table S1 in File S1). We scored \{Ud\}86 allele presence in each generation using the dominant fluorescence of the 3x-P3-RFP marker. Figure 2a shows a consistent and rapid rise in the frequency of \{Ud\}86 when above a threshold frequency estimated at \( p = 0.61 \) and a corresponding decline when below it. The elimination of either + or \{Ud\}86 based on initial frequencies demonstrates the inherent reversibility of underdominant population transformation and also underlines the spatially self-limiting nature of underdominant population transformation. Using a maximum-likelihood approach the relative fitness of the two genotypes relative to a “wildtype”-proxy fitness defined as one was estimated (Figure 2b and c) as \( e^{\lambda \{Ud\}86} = 0.22 \) (0.16–0.28, 95% CI) for the heterozygote and \{Ud\}86/\{Ud\}86 = 0.71 (0.62–0.81, 95% CI) for the homozygote. These fitness values represent a strongly underdominant system. If the \{Ud\}86 allele is pushed above 61% in a population by transgenic releases, it is predicted to proceed to fixation.

Note that the observed egg-to-adult viability reduction alone is insufficient to fully explain the 70–80% reduction in fitness over the entire lifecycle relative to homozygotes (Figure 2b). Given the fundamental importance of protein synthesis to most metabolic processes we speculate that it is likely that the unattributed fitness reduction is the consequence of a large number of small pleiotropic effects. This is consistent with the multiple but generally subtle phenotypes reported for most CRP hypomorphs in Drosophila [12,30,31].

Confirming \( Rpl14^{+4} \) expression and \( Rpl14^{+4} \) knock-down

Estimates of total \( Rpl14 \) mRNA levels were performed using quantitative RT-PCR to establish that: (1) the RNAi knock-down was effective and substantially dominant; (2) that there was significant expression of the rescue. As anticipated, we observed a locus-specific RNAi induced reduction in wildtype \( Rpl14^{+4} \) mRNA in both transgenic genotypes (Figure 3) and substantial expression of the rescue \( Rpl14^{+4} \) (Figure 3 and S7). Given that \( Act5C-GAL4 \) driven expression of a single \( Rpl14 \) dsRNA gene in the absence of any \( Rpl14^{+4} \) gene resulted in 100% lethality (data not shown, see also materials and methods) the generation of near wildtype numbers of viable offspring (Figure S4b in File S1) demonstrated that rescue expression was highly effective.

Discussion

Currently, there are only three population transformation systems that have worked in laboratory experiments: a maternal poison-rescue system termed Medea [32], a homing-endonuclease base system termed HEG [33], and a different type of engineered underdominance based on reciprocal knock-down rescue termed UDMEI [34]. Neither Medea nor HEG achieved complete fixation in experimental populations, with the stable equilibrium frequency of wildtype alleles remaining at approximately 0.1 for Medea (Figure 1F in [34]) and >0.1 for HEG (Figure S5 in [33]). Complete fixation was achieved for UDMEI and {Ud}86 (Figure 2b). A potential concern is that persisting wildtype alleles could facilitate selection for resistance by the insect to the driving properties of HEG or Medea, or selection for resistance by the pathogen to the linked disease refractory gene [35]. While it is possible that future developments of these systems could address this issue [36] it is noteworthy that the underdominant approach demonstrated here reduces the probability of this complication by rapidly eliminating all wildtype alleles within a population (Figure 2a). However, while this does not entirely eliminate this concern; two additional areas warrant future experimental attention. The first is selection for resistance to \( \{Ud\} \), e.g. by genomic duplication or up-regulation of the targeted haploinsufficient gene. The second, which is specific to the targeting of CRP genes, is the potential impact that high frequency viral infections could have in increasing the fitness of \( \{Ud\} \) heterozygotes. This possibility is raised by the finding that infections of the Drosophila C virus (DCV) are inhibited by knocking-down many CRP genes [37]. If future experiments validate either concern, both could potentially be ameliorated to a significant extent through: 1) selecting promoters for knock-down expression which maximize the fitness of transgenic homozygotes; 2) simultaneous targeting of more than one haploinsufficient gene in a single \( \{Ud\} \) construct, one of which is not a CRP gene (e.g. see Table 4 in [38]). The range of possible genotype configurations in the alternative engineered underdominance approach of UDMEI allow for much lower population transformation thresholds, but at the cost of geographic stability and reversibility.

The initiation of underdominant population transformation is undoubtedly a more resource intensive approach than for HEG [33] or Medea [32], where very small numbers of individuals can be released. For our \{Ud\}86 example this would require releases to exceed an allele frequency of 61% in a given wild population.
Consequently, an underdominant approach would probably be impractical if transformation of a large number of substantially isolated populations was required, or if populations were composed of multiple hybridizing sub-species (e.g. *Anopheles gambiae*). In such circumstances, the increased potential of *Medea* and HEG to readily spread between populations and subspecies would be much more efficient. However, where spatial control is valued, Figure 4 illustrates the extent of the geographic stability {Ud}86 could exhibit even with substantial levels of migration [7] (see also File S1).

Obvious candidates for {Ud} transformation include the mosquitoes *Aedes aegypti* [39], *Culex quinquefasciatus* [40], and *Anopheles stephensi* [41], where refractory genes have been developed for dengue fever [42], avian malaria [43], and human malaria [44,45].

Implementation of the UD*MEI* approach in non-model pest species requires the identification and characterization of maternal and zygote expressed genes and promoters within the right range of expression and fitness effects. In contrast, the facts that *RpL*14 was the first gene selected for the development of this {Ud}86 technique, and Act5c the first driver of the RNAi expression

![Figure 3. Genotypic levels of total *RpL14* mRNA expression in groups of whole individuals.](image)

Amount of *RpL14* mRNA in adults and larvae relative to three normalization genes. Height of bars indicate total amount of *RpL14* mRNA based on SYBR green-based quantitative reverse-transcription PCR and each bar is split to represent the proportion of total *RpL14* expressed from the *RpL14* gene in {Ud}86 (white) and the endogenous *RpL14* gene (gray), based on gene-specific TaqMan probes. Error bars represent 1 standard error for three biological replicates. doi:10.1371/journal.pone.0097557.g003

![Figure 4. Geographic Stability.](image)

Interconnected populations are represented as circles, (red wildtype and green {Ud}86 transformed population). If a single target population is transformed (center) migration can result in two undesired transitions; collapse of population transformation (left), or spread (right). We assumed a migration rate of 0.065 every generation and the fitness configuration described in Figure 2b. Then collapse is only likely to occur if the allele frequency of {Ud}86 drops below \( p = 0.77 \). Spread is only likely to occur if {Ud}86 reaches \( p > 0.53 \) frequency in an adjacent wildtype population.

doi:10.1371/journal.pone.0097557.g004
chosen, suggest that our approach may be readily applied in other non-model organisms. The haploinsufficiency and the deleterious nature of CRP mutations is well conserved across wide evolutionary distance with examples in yeast, Arabidopsis, Drosophila, zebrafish, humans and mice (see references in [12,14]). Consequently, CRPs should represent a rich source of genes that can potentially be targeted by {Ud} constructs in any sexually reproducing organism. While there has been no empirical demonstration of linking a disease refractory gene to a population transformation system, it is notable that due to the larger than 3 fold fitness reduction of +/{Ud}86 heterozygotes, relative to {Ud}86/{Ud}86 homozygotes (Figure 2b), it is expected that {Ud} would be well suited to fixing even strongly deleterious linked refractory genes [35]. Furthermore, it is anticipated that the spatial controllability and complete reversibility of {Ud} population transformation would prove a major advantage in attracting public and regulatory acceptance of this type of self-sustaining technology [1,2,32–34].

Further work remains to be done to more fully characterize this system. We suspect it is likely that the majority of the fitness reduction in heterozygotes is directly due to RpL14 haploinsufficiency (because this is a general property of CRPs). However, we do not know what patterns of RNAi expression are sufficient in generating the reduction of RpL14 level. This can be investigated by substituting various GAL4 drivers into the {Ud}86 background by standard crosses. There may also be a fitness contribution from the knockdown of snoRNAs. snoRNAs are small nuclear RNAs that play a role in mediating chemical modification of other RNA molecules [46] and are also expressed from the RpL14 transcript that exist in the transcript’s introns. A number of CRPs described as haploinsufficient Minute loci also contain snoRNAs [47] and some of these snoRNAs are known to modify ribosomal RNA. Eventually, a more complete picture of what is driving CRP loci haploinsufficiency—via ribosomal interactions—might also include quantifying snoRNA abundance, and/or direct RNAi targeting of snoRNAs.

In the configuration reported here the RNAi knock-down gene was placed under GAL4/UAS control split across two chromosomes. This was done to permit the study of the system using different expression patterns and as a fail-safe for initial testing. However, the use of a two-locus GAL4/UAS system is not theoretically integral to our {Ud} approach. A GAL4 driver could be combined into the {Ud} construct, e.g., to generate {RpL14[Δ], UAS-RpL14:dsRNA, Act5C-GAL4}. Alternatively the use of the GAL4/UAS system could be dispensed with entirely, e.g., by generating {RpL14[Δ], Act5C -RpL14:dsRNA}. The complete elimination by replacement of the UAS promoter with the promoter used in a GAL4 driver is unlikely to result in a qualitative change in expression of the RNAi knock-down gene beyond a potential increase in homozygotes (due to having two alleles rather than one RpL14:dsRNA allele). Note that if it did prove problematic to recover homozygous stocks from initially heterozygous individuals (due to haploinsufficiency induced mortality) resulting from embryonic injections, it would be straightforward to inject a stock previously transformed with a co-dominantly marked ‘rescue only’ construct. This stock could then be segregated away in {Ud} homozygotes. However, given that heterozygotes are neither sterile nor inviable this is probably unnecessary for the insertion site examined (see Figure S4 in File S1).

The system presented here demonstrates a significant development in a population transformation system. The system has the potential to be portable not only in insects but across a wide range of sexually reproducing eukarotic organisms. Furthermore, alternative dominant gene-knockout approaches to RNAi such as site-specific nucleases (CRISPRs, TALENs or ZFNs) could also potentially be utilized. However, before this can be used in truly wildtype populations the GAL4/UAS or equivalent system needs to be incorporated into a single loci or removed and replaced with an appropriate direct promoter.

Supporting Information

File S1 Supplementary information file containing Figures S1–S7, Tables S1–S3 and supplementary equations. (DOCX)

Acknowledgments

We thank Kevin Cook for his advice on CRP mutations, Fred Gould for suggesting RNAi targeting, Vanessa L. Reed, Alexander W. E. Franz, and two anonymous reviewers for comments on the manuscript.

Author Contributions

Conceived and designed the experiments: RGR FAR PMA JAD JB. Performed the experiments: RGR FAR PMA JAD JB. Analyzed the data: RGR FAR PMA JAD JB. Contributed reagents/materials/analysis tools: FAR. Wrote the paper: RGR FAR PMA JAD JB. Designed the population experiments: FAR RGR. Performed the population experiments: FAR RGR PMA JAD. Developed the spatial stability analysis: PMA FAR. Developed the allele specific expression assay: JB. Wrote the additional file 1 along with the other authors: JAD. Conceived the design of the underdominant approach and executed the construction of (Ud): RGR. Participated in the in vivo validation of (Ud) construct, measured life history data and performed MLE data analysis: FAR.

References

1. Curtis C (1968) Possible use of translocations to fix desirable genes in insect pest populations. Nature 218: 368–369.
2. Sulkowska SP, Gould F (2006) Gene drive systems for insect disease vectors. Nature 7: 437–445. doi:10.1038/nrg1870
3. Davis S, Bax N, Grewe P (2003) Engineered underdominance allows efficient and economical introgression of traits into pest populations. J Theor Biol 212: 83–90. doi:10.1016/j.jtbi.2001.2357
4. Altrock PM, Traulsen A, Reeves RG, Reed FA (2010) Using underdominance to bi-stably transform local populations. J Theor Biol 267: 62–75. doi:10.1016/j.jtbi.2010.08.004
5. Marshall JM, Hay BA (2012) Confinement of gene drive systems to local populations: A comparative analysis. J Theor Biol 294: 155–171. doi:10.1016/j.jtbi.2011.10.032
6. Hartl DL, Clark AG (1997) Principles of Population Genetics. Sinauer AD, editor Sinauer Associates.
7. Altrock PM, Traulsen A, Reeves RG, Reed FA (2011) Stability Properties of Underdominance in Finite Subdivided Populations. PLoS Comput Biol 7: e1002260. doi:10.1371/journal.pcbi.1002260.
8. Barton N (1979) The dynamics of hybrid zones. Heredity (Edinb) 43: 341–359.
9. White MJD (1978) Modes of speciation. San Francisco: W. H. Freeman and Company.
10. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401–415.
11. Duffy JB (2002) GAL4 system in Drosophila: a fly geneticist’s Swiss army knife. Genesis 34: 1–15. doi:10.1002/gene.10150
12. Marygold SJ, Roote J, Reuter G, Lambertsson A, Ashburner M, et al. (2007) The ribosomal protein genes and Minute loci of Drosophila melanogaster. Genome Biol 8: R216. doi:10.1186/gb-2007-8-10-r216
13. Saebøe-Larssen S, Urbanscyz Molebi B, Lambertsson A (1997) The Drosophila ribosomal protein L14-encoding gene, identified by a novel Minute mutation in a dense cluster of previously undescribed genes in cytogenetic region 60D. Mol Gen Genet 255: 141–151.
14. Kim D-U, Hayles J, Kim D, Wood V, Park H-O, et al. (2010) Analysis of a genome-wide set of gene deletions in the fruit yeast Schizosaccharomyces pombe. Nax Biotechnol 28: 617–623. doi:10.1038/nbt.1629
15. Ito K, Awano W, Suzuki K, Hiromi Y, Yamamoto D (1997) The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurons and glial cells. Development 771: 761–771.
16. Enerly E, Mikkelsen OL, Lyamouri MAY, Lambertsson A (2003) Evolutionary profiling of the U 49 snoRNA gene. 79: 73–79.
17. Bischof J, Macla RK, Heigel M, Karch F, Basler K (2007) An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc Natl Acad Sci U S A 104: 3312–3317. doi:10.1073/pnas.0611531104
18. Seager RD, Ayala FJ, Marks RW (1982) Chromosome interactions in Drosophila melanogaster. II. Total fitness. Genetics 102: 485–502.
19. Ràmia M, Librado P, Casillas S, Rozas J, Barbudilla A (2012) PopDrowser: the Population Drosophila Browser. Bioinformatics 28: 595–596. doi:10.1093/bioinformatics/bts691
20. Williams K (1976) The failure of Pearson’s goodness of fit statistic. 25: 49.
21. Zar J (1999) Biostatistical Analysis. 4th ed. New York: Prentice Hall.
22. Sokal R, Rohlf F (1981) Biometry: the principles and practice of statistics in biological research. New York: Freeman.
23. Clark AG, Feldman MW, Christiansen FB (1981) The estimation of epistasis in components of fitness in experimental populations of Drosophila melanogaster I. A two-stage maximum likelihood model. Heredity (Edinb) 46: 321–346.
24. Wright S (1931) Evolution in mendelian populations. Genetics 16: 97–139.
25. Wilks SS (1935) The likelihood test of independence in contingency tables. Ann Math Stat 6: 190–196.
26. Wilks S (1938) The Large-Sample Distribution of the Likelihood Ratio for Testing Composite Hypotheses. Ann Math Stat 9: 60–62.
27. Ott RL (1993) An Introduction to Statistical Methods and Analysis. 4th ed. Belmont, California: Duxbury Press.
28. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3.
29. Chintapalli VR, Wang J, Dow JAT (2007) Using FlyAtlas to identify better synthetic homing endonuclease-based gene drive system in the human malaria mosquito. Nature 473: 212–215.
30. Marygold SJ, Coelho CMA, Leevers SJ (2005) Genetic Analysis of RpL38 and RpL5, Two Minute Genes Located in the Centric Heterochromatin of Drosophila melanogaster. 695: 683–695. doi:10.1534/gad.104.034124
31. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3.
32. Clark AG, Feldman MW, Christiansen FB (1981) The estimation of epistasis in components of fitness in experimental populations of Drosophila melanogaster I. A two-stage maximum likelihood model. Heredity (Edinb) 46: 321–346.
33. Wright S (1931) Evolution in mendelian populations. Genetics 16: 97–139.
34. Akbari OS, Matzen KD, Marshall JM, Huang H, Ward CM, et al. (2013) A Synthetic Gene Drive System for Local, Reversible Modification and Suppression of Insect Populations: 671–677.
35. Scott TW, Talbot W, Knols B (2002) The ecology of genetically modified mosquitoes. Science (80-) 298: 117–119. doi:10.1126/science.298.5590.117
36. Ito J, Ghosh A, Moreira LA, Wimmer EA (2002) Transgenic anopheles mosquitoes impaired in transmission of a malaria parasite. Nature 417.
37. Cherry S, Doekas T, Armknecht S, Whelan S, Wang H, et al. (2005) Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to host translation inhibition. Genes Dev 19: 445–452. doi:10.1101/gad.1827905
38. Cook RK, Christensen SJ, Deal JA, Coburn RA, Deal ME, et al. (2012) The generation of chromosomal deletions to provide extensive coverage and subdivision of the Drosophila melanogaster genome. Genome Biol 13: R21. doi:10.1186/gb-2012-13-5-r21
39. Kokoza V, Ahmed A, Wimmer EA, Raikhel AS (2001) Efficient transformation of the yellow fever mosquito Aedes aegypti using the piggyBac transposable element vector pBac[SxP5-EGFP afm]. Insect Biochem Mol Biol 31: 1137–1143. doi:10.1016/S0965-7448(01)00320-5
40. Allen M, O’Brochta D, Atkinson P (2001) Stable, germ-line transformation of the Culex quinquefasciatus (Diptera: Culicidae). J Med Entomol: 701–710.
41. Nolan T, Bowser T, Brown A, Criani A (2002) piggyBac-mediated germline transformation of the malaria mosquito Anopheles stephensi using the red fluorescent protein dRED as a selectable marker. J Biol Chem 277: 8759–8762. doi:10.1074/jbc.C100766200
42. Franz AWE, Sanchez-Vargas I, Adelman ZN, Blair CD, Beatty BJ, et al. (2006) Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified Aedes aegypti. Proc Natl Acad Sci U S A 103: 4198–4203. doi:10.1073/pnas.0600479103
43. Jasinskaire N, Coleman J, Ashikyan A, Salampessy M, Marinotti O, et al. (2007) Genetic control of malaria parasite transmission: threshold levels for infection in an avian model system. Am J Trop Med Hyg 76: 1072–1078.
44. Dong Y, Das S, Cirimotich C, Souza-Neto J a, McLean KJ, et al. (2011) Engineered anopheles immunity to Plasmodium infection. PLoS Pathog 7: e1002458. doi:10.1371/journal.ppat.1002458
45. Isaacs AT, Li F, Jasinskaire N, Chen X, Nirmala X, et al. (2011) Engineered anopheles immunity to Plasmodium infection. PLoS Pathog 7: e1002458. doi:10.1371/journal.ppat.1002458
46. Weinstein LB, Steitz JA (1999) Guided tours: from precursor snoRNA to functional snoRNP. Curr Opin Cell Biol 11: 378–384.
47. Holley CL, Topkara VK (2011) An introduction to small non-coding RNAs: miRNA and snoRNA. Cardiovasc drugs Ther 25: 151–159.