Experimental observation of overdominance (heterozygous advantage) in *Drosophila melanogaster*.

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Natural selection is usually assumed to increase the frequency of an advantageous allele or to decrease the frequency of a deleterious allele over time. There are mechanisms that can maintain genetic variation over time, such as cycling environments that favor one allele for a time and then the other and frequency dependent selection, where rare alleles are selected over the more common allele (see Hedrick, 2005, for a discussion of these selection topics).

Fisher (1922) first noted that a stable polymorphism for a pair of alleles of a gene would also occur if the heterozygotes (A_1A_2) were more fit than either of the homozygotes (A_1A_1 and A_2A_2). Fisher (1922) stated:

> "From a consideration of the stability of the frequency ratios, however, it appears that there will only be stable equilibrium if the heterozygote is favoured by selection against both the homozygotes."

This type of selection has been called overdominance, heterozygous advantage, or balancing selection.

At one time it was thought by many that overdominance was very common in natural populations and that this was the reason why so much genetic variation was observed in nature (see discussions of this topic in Dobzhansky, 1955; Hartl and Clark, 2006; Hedrick, 2005; Lewontin, 1974, 1987; Beatty, 1987; Crow, 1987; Paul, 1987). Others, such as H. J. Muller (1950), believed that most genes in nature would be homozygous wild type, since the majority of new mutations are deleterious and would be eliminated by selection. Frequent overdominance began to seem unrealistic when it was noticed that if superior heterozygotes were present in a population, so would the less fit homozygotes. Based on the frequencies of heterozygotes in *Drosophila pseudoobscura*, Lewontin and Hubby (1966) stated:

> "For example, suppose two alleles are maintained by selecting against both homozygotes to the extent of 10% each. Since half of all individuals are homozygotes at such a locus, there is a loss of 5% of the population's reproductive potential because of the locus alone. If our estimate is correct that one third of all loci are polymorphic, then something like 2,000 loci are being maintained polymorphic by heterosis. If the selection at each locus were reducing population fitness to 95% of maximum, the population's reproductive potential would be only $(.95)^{2000}$ of its maximum or about 10^{-46}. If each homozygote were 98% as fit as the heterozygote, the population's reproductive potential would be cut to 10^{-9}. In either case, the value is unbelievably low. While we cannot assign an exact maximum reproductive value to the most fit multiple heterozygous genotype, it seems quite impossible that only one billionth of the reproductive capacity of a Drosophila population is being realized. No Drosophila female could conceivably lay two billion eggs in her lifetime."

This segregation load, plus Haldane's cost of natural selection, which proposed that for a new beneficial allele to go to fixation many individuals would arise that are less fit than the homozygotes with the new beneficial allele (Haldane, 1957; Woodruff *et al.*, 2004), led Motoo Kimura to propose his neutral theory of evolution, where most new mutations, especially at the molecular level, are neutral, *i.e.*, the fitness of genotypes with these alleles are the same (the fitness of $A_1A_1 = A_1A_2 = A_2A_2$) (Kimura, 1983).

There are several examples of overdominance (see review in Gemmell and Slate, 2006). The most famous is the balance of hemoglobin alleles, where humans that are homozygous Hb^AHb^A get malaria, those that are Hb^SHb^S get sickle-cell anemia, but heterozygotes (Hb^AHb^S) do not have sickle-cell anemia and are resistant to the malaria parasite (Allison, 1954, 1956; Hedrick, 2005). A second example is warfarin resistance in rats. Homozygotes are either killed by warfarin or have a vitamin K deficiency, whereas heterozygotes are resistant to warfarin and have normal levels of vitamin K (Greaves *et al.,* 1977). Others, however, support directional selection instead of overdominance (see references in Gemmell and Slate, 2006). A third example is overdominance for plumage color in the common buzzard (*Buteo buteo*) in Germany, where heterozygotes for intermediate color are more fit than the homozygous dark or light morphs (Kruger *et al.*, 2001). Sand shrimp (*Gammarus insensibilis*) heterozygotes for glucosephosphate isomerase also survive better at higher temperatures than either of the homozygotes (Patarnello and Battaaglia, 1992). Female pigeons heterozygous for two alleles of the transferring protein gene have a higher percentage of hatched eggs than either homozygote (Freleger, 1972). Finally, sheep heterozygous for the *BMP15* and *GDF9* genes have increased ovulation rates and litter sizes above those of homozygotes (Gemmell and Slate, 2006).

The reason a polymorphism for a pair of alleles (A_1 and A_2) is maintained by overdominance can be seen in the following one-locus, two-allele model of a population that is assumed to be in Hardy/Weinberg equilibrium except for selection. In this model:

1) s is the selection coefficient against the A_1A_1 genotype (fitness, $w = 1 - s$),
2) t is the selection coefficient against the A_2A_2 genotype (fitness, $w = 1 - t$),
3) the fitness of the A_1A_2 heterozygotes is set at one (fitness, $w = 1$),
4) the frequency of the A_1 allele is p,
5) the frequency of the A_2 allele is q,
6) the mean fitness, which is the sum of the relative contributions of all genotypes, is given the symbol ϖ,
7) the frequency of the A_1 allele at equilibrium is p_e,
8) the frequency of the A_2 allele at equilibrium is q_e.

The following is one possible derivation of the equations for the frequency of the A_1 allele at equilibrium (p_e) and the A_2 allele at equilibrium (q_e). The expected frequencies of q and p at equilibrium with overdominance are:

$q_e = s/(s + t)$ and $p_e = t/(s + t)$, as shown below.

Derivation of the expected overdominance equilibrium frequencies:

	A_1A_1	+	A_1A_2	+	A_2A_2
Frequency	p^2		$2pq$		q^2
Relative fitness =	$1 - s$		1		$1 - t$
After selection =	$p^2(1-s)/\varpi$		$2pq(1)/\varpi$		$q^2(1-t)/\varpi$

Where ϖ $=$ $p^2(1-s) + 2pq(1) + q^2(1-t)$

ϖ $=$ $p^2 - sp^2 + 2pq + q^2 - tq^2$

since $p^2 + 2pq + q^2 = 1$

ϖ $=$ $1 - sp^2 - tq^2$

The expected frequency of q in the next generation (q^1) is:

$$q^1 = \frac{(1/2)(2pq) + q^2(1-t)}{\varpi}$$

$$q^1 = \frac{pq + q^2 - tq^2}{\varpi}$$

Since $pq = (1-q)(q) = q - q^2$

$$q^1 = \frac{q - q^2 + q^2 - tq^2}{\varpi}$$

$$q^1 = \frac{q - tq^2}{\varpi} = \frac{q(1-tq)}{1 - sp^2 - tq^2}$$

The equation for the change in q in one generation (Δq) is determined next, allowing for Δq to then be set equal to zero, for equilibrium, and to solve for p_e and q_e.

$$\Delta q = q^1 - q$$

$$\Delta q = \frac{q(1-tq)}{1 - sp^2 - tq^2} - q$$

$$\Delta q = \frac{q(1-tq) - q(1 - sp^2 - tq^2)}{1 - sp^2 - tq^2}$$

$$\Delta q = \frac{q - tq^2 - q + sp^2 q + tq^3}{1 - sp^2 - tq^2}$$

$$\Delta q = \frac{-tq^2 + sp^2 q + tq^3}{1 - sp^2 - tq^2}$$

Since $q^3 = q^2(1-p)$

$$\Delta q = \frac{-tq^2 + sp^2 q + t[q^2(1-p)]}{1 - sp^2 - tq^2}$$

$$\Delta q = \frac{-tq^2 + sp^2 q + tq^2(1-p)}{1 - sp^2 - tq^2}$$

$$\Delta q = \frac{-tq^2+sp^2q+tq^2-tpq^2}{1-sp^2-tq^2}$$

$$\Delta q = \frac{sp^2q-tpq^2}{1-sp^2-tq^2}$$

$$\Delta q = \frac{pq(sp-tq)}{1-sp^2-tq^2}$$

We next want to determine the frequency of q_e (q at equilibrium), when q and p do not change. To do this, set the change in q, Δq, equal to zero.

At equilibrium:

$$\Delta q = 0$$

$$\Delta q = \frac{pq(sp-tq)}{1-sp^2-tq^2} = 0$$

$$pq(sp-tq) = 0$$

$$sp - tq = 0$$

$$s(1-q)-tq = 0$$

$$s-sq-tq = 0$$

$$-sq-tq = -s$$

$$q(-s-t) = -s$$

$$q_e = -s / -s -t$$

$$q_e = s / s + t$$

and since $p_e = 1 - q_e$

$$p_e = 1 - (s / s + t)$$

$$p_e = s + t - s / s + t$$

$$p_e = t / s + t$$

In summary, with overdominance the expected frequencies of p_e and q_e can be determined from the selection coefficients against the A_1A_1 (s) and A_2A_2 (t) genotypes, with the equilibrium frequency of q being equal to s/(s + t) and the equilibrium frequency of p being equal to t/(s + t).

An example of overdominance is shown in Figure 1, where s and t are equal to 0.5 and the original value of q ranges from 0.1 to 0.9. After a few generations, q and p are at equilibrium and are equal to 0.5. This figure was generated from the AlleleA1 program of Jon C. Herron (see his web site, http://faculty.washington.edu/herronjc/SoftwareFolder/AlleleA1.html).

We attempted to observe overdominance in two sets of crosses based on experiments by Provine (1971) using the fourth-chromosome visible markers eyeless-2 (ey^2) and shaven-naked (sv^n), and by using a combination of a second chromosome marked with a recessive visible phenotype (white eyes) and a second chromosome containing a large inversion and a recessive lethal mutation, similar to the experiment by Mukai and Burdick (1959).
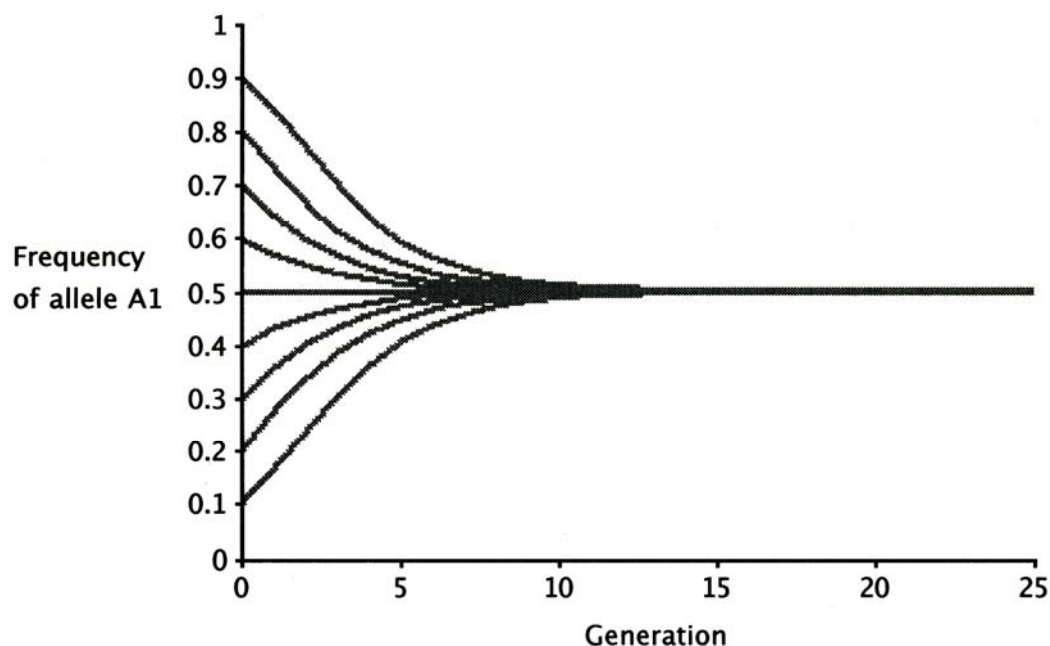
Figure 1. The equilibrium values for the expected overdominance values of $q_e(A1) = s/(s + t)$, with $s = 0.5$, $t = 0.5$, and q from 0.1 to 0.9.

Overdominance using the eyeless (ey^2) and shaven-naked (sv^n) fourth-chromosome mutations

Provine (1971) observed that heterozygotes for ey^2 (reduced eyes) and sv^n (reduced bristle size), *i.e.*, $ey^2 + / + sv^n$ flies, have a higher fitness (viability) than either of the homozygotes ($ey^2 + / ey^2 +$ or $+ sv^n / + sv^n$). It should be noted that since recombination does not occur for fourth-chromosome genes in *D. melanogaster* the eyeless and shaven alleles do not recombine and $ey^2 +$ and $+ sv^n$ can be considered as constant haplotypes. For these three genotypes Provine (1971) observed:

		$ey^2 + / ey^2 +$	$ey^2 + / + sv^n$	$+ sv^n / + sv^n$
Relative fitness	=	1- s	1	1 - t
Relative fitness	=	0.6	1	0.4
Selection coefficient	=	0.4		0.6

Based on overdominance theory, the frequency of $ey^2 +$ (p_e) at equilibrium was expected to be:

$$p_e = t / s + t = 0.6 / (0.4 + 0.6) = 0.6.$$

Provine (1971) observed that with a beginning frequency of $ey^2 + / + sv^n$ at 100 percent, the final equilibrium frequencies of $ey^2 +$ in five bottles after 25 generations were: 0.58, 0.51, 0.59, 0.55, and 0.57. All values were close to the expected value of 0.6. In addition, if the frequency of $ey^2 +$ at the beginning was below or above 0.6, the frequency of $ey^2 +$ moved over time to about 0.6 (Provine, 1971). These results supported the expectations from overdominance theory.

We repeated the Provine (1971) experiment by first testing for progeny viability from crosses of 50 $ey^2 + / + sv^n$ females and 50 $ey^2 + / + sv^n$ males in four ½-pint milk bottles at 25°C. The $ey^2 + / ey^2 +$ and $+ sv^n / + sv^n$ flies used to generate these heterozygotes were obtained from Indiana

University (stocks numbers 648 and 663, respectively). The numbers of progeny from these crosses were:

	$ey^2 + / ey^2 +$	$ey^2 + / + sv^n$	$+ sv^n / + sv^n$
Bottle			
1	101	318	98
2	63	198	34
3	116	337	81
4	147	411	81
Total	427	1,264	294

This gives the following viability results (the number of heterozygotes is divided by two, because the expected Mendelian results are a 1:2:1 ratio):

	$ey^2 + / ey^2 +$	$ey^2 + / + sv^n$	$+ sv^n / + sv^n$
Proportion of progeny =	427/1353	632/1353	294/1353
Proportion of progeny =	0.316	0.467	0.217

Hence, the $ey^2 + / ey^2 +$ homozygotes are more fit (more viable) than the $+ sv^n / + sv^n$ homozygotes, and the $ey^2 + / + sv^n$ heterozygotes are more fit than either of the homozygotes (overdominance). Setting the fitness of the heterozygotes to one (0.467/0.467 = 1) gives:

		$ey^2 + / ey^2 +$	$ey^2 + / + sv^n$	$+ sv^n / + sv^n$
Relative fitness	=	0.316/0.467	0.467/0.467	0.217/0.467
Relative fitness	=	0.68	1	0.47
Selection Coefficient =		s = 0.32		t = 0.53

The expected equilibrium frequency of the $ey^2 +$ haplotype (p_e), based on overdominance theory, is:

$$p_e = t / s + t = 0.53 / (0.32 + 0.53) = 0.62.$$

This value is very close to the previous value of Provine (1971) of 0.60.

Based on the expected equilibrium value of 0.62 for $ey^2 +$, we set up three bottles with $ey^2 +$ at a frequency of 0.75 by mating 30 virgin $ey^2 + / + sv^n$ females with 30 $ey^2 + / ey^2 +$ males (the frequency of ey^2 = 90/120 = 0.75) and three bottles with $ey^2 +$ at a frequency of 0.25 by mating 30 $ey^2 + / + sv^n$ virgin females with 30 $+ sv^n / + sv^n$ males (ey^2 = 30/120 = 0.25). We predicted that the frequency of $ey^2 +$ in these bottles will, with time, move toward the expected equilibrium frequency of 0.62 (see Figure 1 for a similar case). All six bottles (three for $ey^2 +$ = 0.75, and three for $ey^2 +$ = 0.25) were then maintained at 25°C, the adults cleared after seven days, and progeny counted for 14 days from time of initiation of the crosses. The progeny from each bottle were stored in a holding bottle until the last count on day 14; all flies were then placed into a new bottle to begin the next generation. This procedure was repeated for a total of six generations. Each generation the numbers of females and males were counted for the three genotypes ($ey^2 + / ey^2 +$ = eyeless flies: $ey^2 + / + sv^n$ = flies with normal eyes and long bristles; $+ sv^n / + sv^n$ = flies with short bristles). It should be noted that no recombinants ($ey^2 sv^n$ or $+ +$) were observed during this study.

The results of this experiment are shown in Figure 2. In all of the 0.75 bottles, by the sixth generation the frequency of $ey^2 +$ decreased to a mean of 0.71 ± 0.01SE, and in all of the 0.25 bottles

the frequency of ey^2 + increased to 0.70 ± 0.03SE.   The average frequency of ey^2 + for all six bottles was 0.71 ± 0.01SE. These results support the overdominant population genetic theory, but gave an equilibrium value for ey^2 + (0.71) that was significantly higher than predicted from the original one-generation experiments (0.62)(P = 0.001). It is not unexpected that the multigenerational value may be different from the one-generation estimation of the equilibrium value of ey^2 +. For example, additional factors may play a role in overall fitness in a mutigenerational experiment, including mating success, fertility, and aging.
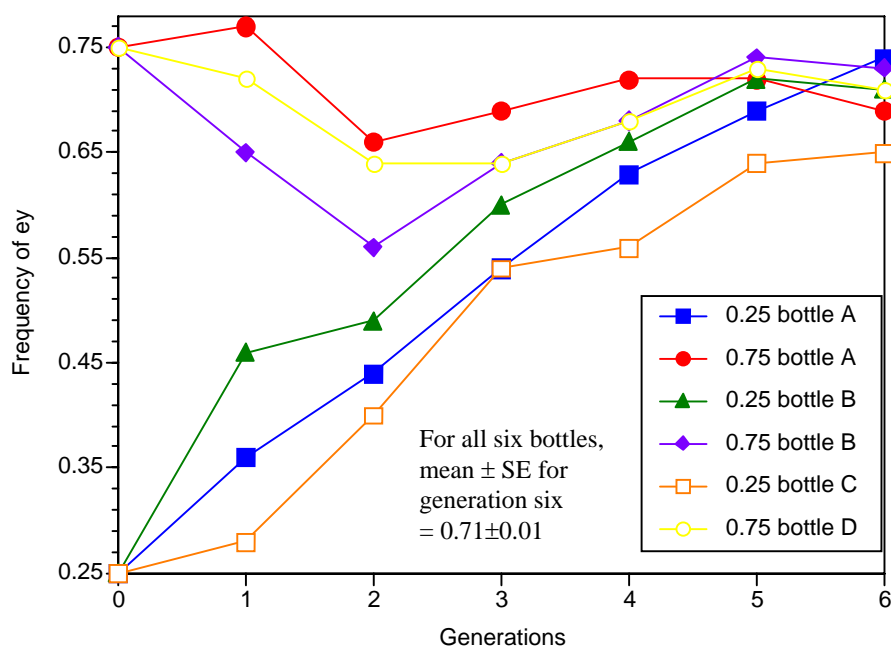
Figure 2. Frequencies of ey^2 + over time in six crosses of ey^2 + / ey^2 + , ey^2 + / + sv^n and + sv^n / + sv^n flies with fitness values of 1-s, 1, and 1-t. Three bottles were started with an ey^2 + frequency of 0.75 and three with an ey^2 + frequency of 0.25. At equilibrium, ey^2 + is expected to be equal to t/(t +s), which here is about 0.71.

Overdominance using a marked (cinnabar and brown = recessive white eyes) second chromosome and an In(2LR)Px^4, dp^{ov1} b second chromosome, which also contained a recessive lethal mutation

Since we observed that the shaven-naked (sv^n) phenotype in the previous study had variable expression that ranged from flies with almost no eyes to flies with almost wild-type eyes, we decided to test a new set of crosses that would allow one to identify overdominance more easily in *D. melanogaster*. We obtained the stock In(2LR)Px^4, *dp b*/CyO from the University of Indiana (Stock # 1473), which is known to contain a recessive lethal mutation in the In(2LR)Px^4 chromosome (Burdick, 1956), and crossed it with a homozygous stock of *cn bw/cn bw* (flies homozygous for these two recessive second-chromosome mutations have white eyes; Lindsley and Zimm, 1992). We then selected for In(2LR)Px^4, *dp b/cn bw* progeny and maintained this stock by selecting for red-eyed parents each generation. In this study, the *cn bw* chromosome and the In(2LR)Px^4, *dp b* chromosome will be treated as two haplotypes. This is appropriate because there are no viable recombination

events separating *cn* from *bw* in In(2LR)Px^4, *dp b/cn bw* heterozygotes, due to the large *Px^4* inversion. From a cross of these flies we expected the following progeny.

$$\text{In(2LR)Px}^4,\ dp\ b/cn\ bw \qquad \times \qquad \text{In(2LR)Px}^4,\ dp\ b/cn\ bw$$

$$cn\ bw/cn\ bw\ \text{(white eyes)} \qquad \text{and} \qquad \text{In(2LR)Px}^4,\ dp\ b/cn\ bw\ \text{(red eyes)}.$$

The In(2LR)Px^4, *dp b*/ In(2LR)Px^4, *dp b* flies do not survive because of a second-chromosome recessive lethal mutation. If they did survive, the flies would have dumpy wings (*dp*) and a black body color (*b*); we did not observe any flies with these phenotypes in this study.

From crosses of In(2LR)Px^4, *dp b/cn bw* females and males, the expected fitness (viability) values for their progeny are (In = In(2LR)Px^4, *dp b*):

	cn bw / cn bw	In / *cn bw*	In / In
Relative Fitness =	1 − s	1	1 − t
			Since t = 1,
Relative Fitness =	1 − s	1	0

To estimate s, t and the expected equilibrium value for *cn bw* (p$_e$), we mated 50 virgin In(2LR)Px^4, *dp b/cn bw* females and 50 In(2LR)Px^4, *dp b/cn bw* males in each of three bottles and counted the progeny. Since the expected Mendelian ratios are 1 *cn bw/cn bw* : 2 In/*cn bw*, we expected twice as many red-eyed progeny from these crosses. Hence, we doubled the number of white-eyed (*cn bw/cn bw*) progeny to estimate the proportion of progeny. The following progeny counts were observed.

	cn bw / cn bw	In / *cn bw*	In/In
Bottle 1	154 x 2 = 308	449	0
Bottle 2	137 x 2 = 274	420	0
Bottle 3	77 x 2 = 154	278	0
Totals	736	1147	0
Proportion of progeny =	736/1883	1147/1883	0/1883
Proportion of progeny =	0.39	0.61	0

The heterozygotes clearly are more viable than the homozygotes.

Setting the fitness of the heterozygotes to one (0.61/0.61 = 1) gives:

	cn bw / cn bw	In / *cn bw*	In / In
Relative fitness =	0.39/0.61 = 0.63	0.61/0.61 = 1	0/0.61 = 0
Selection coefficient =	s = 0.37		t = 1

The expected equilibrium frequency of the *cn bw* haplotype (p$_e$), based on overdominance theory is:

$$p_e = \ t\ /\ s + t\ =\ 1\ /\ (0.37\ +\ 1)\ =\ 0.73$$

Based on the expected equilibrium value for *cn bw* (p_e) of 0.73, we set up four bottles with 50 virgin In(2LR)Px^4, *dp b/cn bw* females and 50 In(2LR)Px^4, *dp b/cn bw* males per bottle for a beginning frequency of 0.50 for *cn bw* (100 *cn bw* alleles and 100 In(2LR)Px^4, *dp b* alleles) and four bottles of 70 virgin *cn bw/cn bw* females with 30 In(2LR)Px^4, *dp b/cn bw* males for a beginning frequency of 0.85 for *cn bw* (170 *cn bw* alleles and 30 In (2LR)Px^4, *dp b* alleles). We predicted that the frequency of *cn bw* in these bottles would, with time, move toward the expected equilibrium frequency of 0.73 (see Figure 1 for a similar case). All eight bottles (four for *cn bw* = 0.50 and four for *cn bw*= 0.85) were then maintained at 25°C, the adults cleared after seven days, progeny counted for 14 days from time of initiation of the crosses, and all flies were placed into a new bottle to begin the next generation. This procedure was repeated for a total of six generations. Each generation the number of females and males were counted for the two genotypes (*cn bw/cn bw* = white-eyed flies and In(2LR)Px^4, *dp b/cn bw* = red-eyed flies). The white and red-eyed mutants had phenotypes with constant expression.
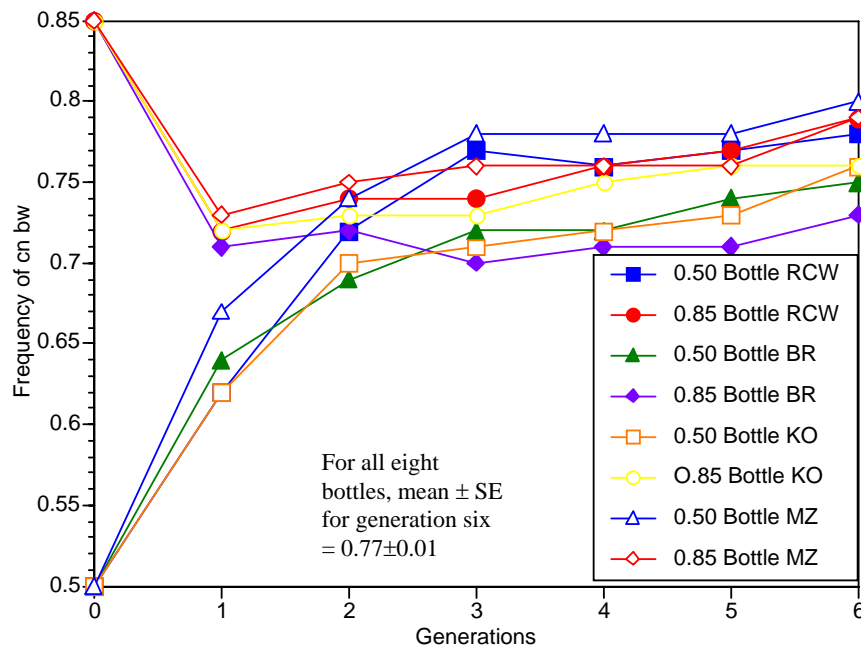

Figure 3. Frequencies of *cn bw* (white eyes) over time in eight crosses of In(2LR)Px^4, *dp^{ov1} b/cn bw* flies with fitness values of 1-s, 1 and 0 for *cn bw/cn bw*, In(2LR)Px^4, *dp^{ov1} b/cn bw* and In(2LR)Px^4, *dp^{ov1} b/* In(2LR)Px^4, *dp^{ov1} b* progeny. Four bottles were started with a *cn bw* frequency of 0.85 and four with a *cn bw* frequency of 0.5. At equilibrium, *cn bw* is expected to be equal to t/(t +s), which here is about 0.77

The results of this experiment are shown in Figure 3. By the sixth generation, in all four of the 0.85 bottles the frequency of *cn bw* decreased to a mean of 0.77 ± 0.01SE, and in all of the 0.50 bottles the frequency of *cn bw* increased up to 0.77 ± 0.01SE. The average frequency of *cn bw* for all eight bottles was 0.77 ± 0.01SE. These results support the overdominant population genetic theory.

As with the previous experiment, however, the observed equilibrium value for *cn bw* (0.77) was significantly higher than the one-generation value of 0.73 (P = 0.002).

Based on the above results, it is expected that the *cn bw* and In(2LR)Px[4], *dp b* chromosomes will be maintained in this population over time by balancing selection. We are currently testing this overdominance hypothesis by maintaining 50 bottles of this experiment over a long period of time. We are also monitoring for increases in the proportion of *cn bw/cn bw* flies in each of these bottles due to selection for new recessive beneficial mutations that occur on the *cn bw* second chromosome. As a control, recessive beneficial mutations occurring on the In(2LR)Px[4], *dp b* chromosome will not become homozygous because this chromosome contains a recessive lethal mutation. In addition, new deleterious recessive mutations will be eliminated in *cn bw/cn bw* flies by selection. Hence, we hypothesize that recessive beneficial mutations will increase the equilibrium value of *cn bw* (p_e) over time.

Finally, based on overdominance theory with one homozygote having a fitness of zero (t = 1), the frequency of *cn bw* (p_e) at equilibrium is expected to be:

$$p_e = t / (s + t)$$

Since t = 1,

$$p_e = 1 / (s + 1)$$

$$p_e(s + 1) = 1$$

$$p_e s + p_e = 1$$

$$p_e s = 1 - p_e$$

$$s = q_e / p_e$$

Hence, if the equilibrium values are known for p and q, one can estimate s. In this experiment, therefore,

$$s = 0.27 / 0.73 = 0.37 \text{ for the } cn\ bw/cn\ bw \text{ homozygotes.}$$

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