Instant synthetic species: tests of sexual isolation between compound-autosome stocks of *Drosophila melanogaster*.

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In the 1960s and 1970s, compound autosome stocks of *Drosophila melanogaster* were synthesized, making these stocks "instant synthetic species". These stocks are considered new species, because when they mate with *D. melanogaster* that have a normal karyotype, no progeny survive. The progeny of these crosses do not survive, because they have extra and missing autosome arms, and, therefore, triple and single copies of genes on these chromosome arms (see reviws of this topic, including the methods to synthesize the compound autosomes, in Holm, 1976; Ashburner, 1989).

In 1960, Rasmussen reported the synthesis of a C(3L)RM; C(3R)RM stock, which has the two left arms of the third chromosome attached to a centromere and the two right arms attached to a centromere (3L . 3L and 3R . 3R, with "." being the centromere), as compared to the left arms being attached to the right arms (3L . 3R and 3L . 3R) in wild-type *D. melanogaster*. Figure 1 shows what is expected when this compound-autosome stock is mated with a stock with normal third chromosomes (also see Figure 25.2, page 784, Ashburner, 1989); no adult progeny are expected to survive. Since the biological species concept is based on organisms being separate species if they are reproductively isolated (Coyne and Orr, 2004), then the C(3L)RM; C(3R)RM stock is an "instant synthetic species".

Figure 1. Expected results of matings between a C(3L)RM; C(3R)RM stock and a stock with normal third chromosomes (3L . 3R).

	C(3L)RM gamete	C(3R)RM gamete	C(3L)RM and C(3R)RM gamete	No third chromosome gamete
3L . 3R gamete	C(3L)RM / 3L . 3R Three 3L and one 3R Flies do not survive	C(3R)RM / 3L . 3R Three 3R and one 3L Flies do not survive	C(3L)RM; C(3R)RM/ 3L . 3R Three 3L and three 3R Flies do not survive	3L . 3R One 3L and one 3R Flies do not survive

Figure 2. Expected results of crosses between a C(2L)RM, F(2R) stock and a stock with normal second chromosomes.

	C(2L)RM; 2R gamete	2R gamete
2L . 2R gamete	C(2L)RM; 2R / 2L . 2R Three 2L Flies do not survive	2R / 2L . 2R One 2L Flies do not survive

As another example of a compound autosome stock, Grell in 1970 reported the synthesis of a C(2L)RM, F(2R) stock, which has two left arms of the second chromosome attached to a centromere and two free right arms of the second chromosome, each with a centromere (2L . 2L, 2R., and 2R.). Wild-type *D. melanogaster* have the left arms of the second attached to the right arms (2L . 2R and 2L . 2R) (also see Figure 1, page 66 of Grell, 1970). Figure 2 shows what is expected when this compound autosome stock is mated with a stock with normal second chromosomes (Boulton and Woodruff, 2010). Again, no adult progeny are expected to survive, also making the C(2L)RM, F(2R) stock an "instant synthetic species".

Based on the dates of their synthesis, the C(3L)RM, *ri*; C(3R)RM, e^{4} and the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stocks that are used in this study have been isolated from normal-karyotype *D. melanogaster* for 53 and 43 years, respectively. Since the generation time of *D. melanogaster* laboratory stocks is about three weeks, the C(3L)RM, *ri*; C(3R)RM, e^{4} stock has been reproductively isolated from other *D. melangaster* flies with normal chromosomes for about 919 generations, whereas the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock has been isolated for about 745 generations. Taken together, the two stocks have evolved separately for 1,664 generations. Has this been enough time for the two stocks to begin to develop reproductive isolation? Do the compound autosome stocks prefer to mate within the same stock more than beween the two stocks?

To begin to answer these questions, we first tested to make sure that the C(3L)RM, *ri*; C(3R)RM, e^{4} and C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stocks have rearranged autosomes, and then measured their sexual isolation (mating preference). The mutant genes in these stocks are: *ri* = radius incompletus (short wing vein); e^{4} = ebony-4 (dark body color); dp^{ov1} = dumpy-ov1 (wings); *c* = curved (wings); *cn* = cinnabar (eye color); *bw* = brown (eye color); *cn* and *bw* together gives a white-eye color to the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock, whereas the C(3L)RM, *ri*; C(3R)RM, e^{4} stock has red eyes (Lindsley and Zimm, 1992).

Table 1. Results of crosses between the compound autosome stocks and Canton-S that has normal second and thrid chromsomes.

Males Females	Canton-S males
C(3L)RM, *ri*; C(3R)RM, e^{4} females	No larva, pupae, or adult progeny were recovered; just unhatched eggs.
C(2L), RM, dp^{ov1}; F(2R), *c cn bw* females	Larvae and pupae were recovered, but no adults. Fitz-Earle and Holm (1978) saw similar results.

Table 2. Expected results from crosses of the two compound autosome stocks.

Males Females	2L . 2R; C(3L)RM; C(3R)RM males	C(2L)RM; F(2R); 3L . 3R males
2L . 2R; C(3L)RM; C(3R)RM females	Some adults are expected to survive	Extra or missing chromosome arms. Flies do not survive
C(2L)RM; F(2R); 3L . 3R females	Extra or missing chromosome arms. Flies do not survive	Some adults are expected to survive

I. Genetic tests of the chromosomal structure of the C(3L)RM, *ri*; C(3R)RM, e^{4}, and C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stocks:

Table 1 shows the results from crosses of the two autosomal stocks with the wild-type Canton-S stock, which has normal second and third chromosomes. No adults survived in either cross. These results confirm that the two compound autosome stocks contain rearranged chromosomes.

Next, the two compound autosome stocks were mated with each other. The expected results (no adult progeny are expected to survive) of these crosses are shown in Table 2, and the observed results are shown in Table 3. As predicted, no adult progeny survied, confirming that the two compound autosome stocks have different rearranged chromosomes.

Table 3. Observed results of matings between the two compound autosome stocks.

Males Females	C(3L)RM, *ri*; C(3R)RM, e^4 males	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* males
C(3L)RM, *ri*; C(3R)RM, e^4 females	Adults survived	No larvae, pupae or adults were recovered.
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* females	No larvae, pupae, or adults were recovered	Adults survived

II. Sexual isolation (mating preference) experiments:

Using a technique similar to Crossley (1974), we performed all pair-wise crosses between females and males of the two compound autosome stocks, first using one female and two males (female choice) and then one male and two females (male choice). For a review of methods for measuring sexual isolation (mating preference) with *D. melanogaster*, see Spieth and Ringo (1983) and Nanda and Singh (2012).

Unetherized three-day-old virgin females and three-day-old males were placed in empty vials and were observed for matings for up to two hours. When a mating was observed, the identification of the mating male (female choice experiment) or mating female (male choice experiment) was determined. The results of these mating experiments are shown in Tables 4 and 5. In Table 4 (female choice), a single C(3L)RM, *ri*; C(3R)RM, e^4 female or a single C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female was mated with two males, (one C(3L)RM, *ri*; C(3R)RM, e^4 male and one C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male), whereas in Table 5 (male choice) a single C(3L)RM, *ri*; C(3R)RM, e^4 male or a single C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male was mated with two females (one C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female and one C(3L)RM, *ri*; C(3R)RM, e^4 female).

Table 4. Female choice sexual isolation (mating preference) experiment, using one female and two males (one from each compound autosome stock).

Males (two) Female (one)	C(3L)RM, *ri*; C(3R)RM, e^4 male	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male
C(3L)RM, *ri*; C(3R)RM, e^4 female	46[a] Number matings if random = 29[a]	12[a] Number matings if random = 29[a]
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female	38[b] Number matings if random = 39[b]	40[b] Number matings if random = 39[b]

[a]P = 0.002; [b]P = 1.

As an example of the results in the upper cross of Table 4, 46 of the 58 total matings were between C(3L)RM, *ri*; C(3R)RM, e^4 flies, and 12 were between C(3L)RM, *ri*; C(3R)RM, e^4 females and C(2L)RM, dp^{ov1}; F(2R), *c cn bw* males. The significant P value of 0.002 comes from a Fisher exact test (using the Prism program) of 46 and 12 observed matings compared to the expected 29 and 29, if the matings were at random. See Dodd (1989) for a similar analysis of reproductive isolation tests of *D. pseudoobscura* strains.

In the female choice experiment (Table 4), the C(3L)RM, *ri*; C(3R)RM, e^4 stock preferred to mate with their own karotype ($P = 0.002$), whereas the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock mated at random with the C(3L)RM, *ri*; C(3R)RM, e^4 and C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stocks ($P = 1$). In the male choice experiment (Table 5), neither stock showed a mating preference ($P = 0.85$ for the C(3L)RM, *ri*; C(3R)RM, e^4 stock and $P = 0.18$ for the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock). Hence, the C(3L)RM, *ri*; C(3R)RM, e^4 stock showed significant sexual isolation (based on the female choice experiment), whereas, the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock mated at random.

As an interspecies control, where matings are expected to occur preferentially among species (Sturtevant, 1920; Ashburner, 1989, p. 1186), we also set up female and male choice mating experiments with the two compound autosome stocks mated with a w^{pch} white-eyed mutant stock of *D. simulans*, a sibling species of *D. melanogaster*. The results of these crosses are shown in Tables 6-9.

Table 5. Male choice sexual isolation (mating preference) experiment, using one male and two females (one from each compound autosome stock).

Males (one) Female (two)	C(3L)RM, *ri*; C(3R)RM, e^4 male	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male
C(3L)RM, *ri*; C(3R)RM, e^4 female	26[a] Number matings if random = 28[a]	8[b] Number matings if random = 14.5[b]
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female	30[a] Number matings if random = 28[a]	21[b] Number matings if random = 14.5[b]

[a]P = 0.85; [b]P = 0.18

In the female and male choice experiments, there is a clear preference for matings within species. For example, from Tables 6-9, there are 65 total matings between *D. simulans* and *D. melanogaster*, 172 matings within *D. simulans* (86 × 2, because there are half as many chances for these mating as matings between species), and 224 matings (112 × 2) within *D. melanogaster*, giving a chi-square value of 50.7 ($P < 0.0001$; 65, 172 and 224 *vs* 153.67, 153.67, and 153.67, if matings were at randaom). In addition, there were significant mating preferences within species in Tables 6 and 7.

One possible reason why the C(3L)RM, *ri*; C(3R)RM, e^4 stock has more sexual isolation than the C(3L)RM, *ri*; C(3R)RM, e^4 flies is because the former stock is more active than the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock. For example, the C(3L)RM, *ri*; C(3R)RM, e^4 males in Table 4 may have increased activity and, therefore, may simply encounter the C(3L)RM, *ri*; C(3R)RM, e^4 females more often than the C(3L)RM, *ri*; C(3R)RM, e^4 males. Hence, locomotion activity was measured for ten males for each of the two compound autosome stocks by use of the Connolly (1966) circular runway apparatus (Woodruff *et al*., 1999). In this apparatus, three-day-old, unetherized, flies were introduced into a 3 mm diameter channel in a 15.7 cm round runway that is marked with radial lines (see Figure 5, Connolly, 1966). Single, unetherized, males were placed into the runway for 90 seconds to recover from the transfer process and then the number of radial lines crossed by the fly in one minute was recorded (distance between lines is 2 cm). The locomotion activities of the two stocks were significantly different ($P = 0.04$). Yet, the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock had a significantly higher locomotion (136.10 ± 10.68 lines per minute; mean ± SE) than the C(3L)RM, *ri*; C(3R)RM, e^4 stock (99.30 ± 12.79). Hence, the increased sexual isolation of the C(3L)RM, *ri*; C(3R)RM, e^4 stock is not due to increased activity.

Table 6. Female choice sexual isolation (mating preference) experiment, using one female and two males (a *D. simulans* male and a compound autosome male).

Males (two) Female (one)	*Drosophila simulans* w^{pch} male	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male
Drosophila simulans w^{pch} female	44^a Number matings if random = 28^a	12^a Number matings if random = 28^a
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female	29^b Number matings if random = 40^b	51^b Number matings if random = 40^b

^{a}P = 0.003; ^{b}P = 0.11

Table 7. Female choice sexual isolation (mating preference) experiment, using one female and two males (a *D. simulans* male and a compound autosome male).

Males (two) Female (one)	*Drosophila simulans* w^{pch} male	C(3L)RM, *ri*; C(3R), e^4 male
Drosophila simulans w^{pch} female	29^a Number matings if random = 20^a	11^a Number matings if random = 20^a
C(3L)RM, *ri*; C(3R), e^4 female	0^b Number matings if random = 11.5^b	23^b Number matings if random = 11.5^b

^{a}P = 0.07; ^{b}P = 0.0002

Table 8. Male choice sexual isolation (mating preference) experiment, using one male and two females (a *D. simulans* female and a compound autosome female).

Males (one) Female (two)	*Drosophila simulans* w^{pch} male	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male
Drosophila simulans w^{pch} female	5^a Number matings if random = 2.5^a	6^b Number matings if random = 12^b
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female	0^a Number matings if random = 2.5^a	18^b Number matings if random = 12^b

^{a}P = 0.17; ^{b}P = 0.14

Table 9. Male choice sexual isolation (mating preference) experiment, using one male and two females (a *D. simulans* female and a compound autosome female).

Males (one) Female (two)	*Drosophila simulans* w^{pch} male	C(3L)RM, ri; C(3R), e^4 male
Drosophila simulans w^{pch} female	8^a Number matings if random = 5^a	5^b Number matings if random = 12.5^b
C(3L)RM, *ri*; C(3R), e^4 female	2^a Number matings if random = 5^a	20^b Number matings if random = 12.5^b

^{a}P = 0.35; ^{b}P = 0.07

In summary, the C(3L)RM, *ri*; C(3R)RM, e^4 stock has more sexual isolation than the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock. The C(3L)RM, *ri*; C(3R)RM, e^4 flies prefer to mate within the stock more than the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock. Furthermore, the mating preference of the C(3L)RM, *ri*; C(3R)RM, e^4 stock is not due to an increase in locomotion. Yet, it is impossible to know if the observed isolation differences have occurred during the 1,664 generations of separation of the two stocks, or were present in the base stocks before they were synthesized into compound autosome stocks. Although most natural population lines of *D. melanogaster* mate at random (Henderson and Lambert, 1982), significant differences in sexual isolation have been observed for laboratory and wild stocks of *D. melanogaster* (for example, Sturtevant, 1915; Wu *et al*., 1995; Haerty *et al*., 2002; Takahashi and Ting, 2004; Alipaz *et al*., 2005).

A class discussion of the results of this teaching exercise could include:

1) For students to see the extent of research on sexual isolation in *Drosophila*, they could go to FlyBase [http://flybase.bio.indiana.edu] and search under references for the key words, sexual isolation, mating preference, and reproductive isolation; they will recover 922 articles with these words in titles or in abstracts from 1938 to 2013.

2) How would larvae and pupae survive from crosses of C(2L), RM, dp^{ov1}; F(2R), *c cn bw* females with Canton-S males, even though the larvae and pupae have extra and missing chromosome arms (see Table 1 and Fitz-Earle and Holm, 1978)? It is known that aneuploidy (missing or extra chromosomes) in humans leads to early embryo death, unless the aneuploidy involves the sex chromosomes, or chromosomes 13, 18, or 21 (Strachan and Read, 2004). Students might consider that triploid *D. melanogaster* with three of each chromosome, survive (Bridges, 1922, Figure 1); there are no triploid humans.

3) Students might be asked to go to FlyBase [http://flybase.bio.indiana.edu] and see if they can find other examples of compound autosomes. For example, there are C(2)EN, C(3)EN, and C(2;3)EN stocks (EN = entire) (Ashburner, 1989). The C(2:3)EN stock has the 2L, 2R, 3L, and 3R chromosome arms attached to one centromere. Novitski *et al*. (1981) reviews the synthesis of the C(2)EN, C(3)EN, and C(2;3)EN chromosomes.

4) One could also discuss other uses of the compound autosome stocks. For example, they have been used to measure fitness of *D. melanogaster* stocks (for reviews of this topic, see Jungen and Hartl, 1979; Sved, 1989). Suppose the intended goal is to measure the fitness of two wild-type

stocks (A and B) that have normal chromosomes. You could not mix the two stocks together and compare offspring production, because the two stocks have the same, wild type, phenotype. Yet, if you place stock A in a bottle with a compound autosome stock that is marked with a visible mutation, you could measure the proportion of A offspring and compound autosome offspring. This progeny ratio could then be compared to the results of B flies and the compound autosome. This would give the fitness of A and B stocks compared to the compound autosome stock and, therefore, the fitness of stocks A and B compared with each other. The compound-autosome technique is a one-generation test of overall fitness, including mating ability, fecundity, fertility, and viability, and has been used to measure the fitness of stocks in the presence and absence of active transposable DNA elements (Belyaeva *et al*., 1982; Woodruff *et al*., 1999) and the fitness of stocks that are resistant or sensitive to insecticides (Minkoff and Wilson, 1992).

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Drosophila smoking: using flies in a smoke-free class.

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Overview

Smoking habit is responsible for 71% of cases of lung cancers, 42% of deaths for chronic respiratory disease, and 10% of cardiovascular diseases (Öberg *et al*., 2010). According to the World