Chapter 12

Inbreeding Depression and the Evolutionary Advantage of Outbreeding

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Chris received a B.Sc. in Zoology at the University of Western Ontario (1982), an M.Sc. in Behavioral Ecology at Carleton University (1985), a Ph.D. in Evolutionary Botany at the University of Toronto (1993), and is currently an Assistant Professor of Biology at Queen's University. His research explores the evolutionary ecology of flowering plants, particularly the evolution of reproductive strategies and the genetic and ecological consequences of colonization. This laboratory exercise was written for the introductory biology course at the University of Toronto, and was successfully completed by approximately 3000 first-year students during the 91/92 and 92/93 academic sessions.

Reprinted from: Eckert, C. G. 1994. Inbreeding depression and the evolutionary advantage of outbreeding. Pages 215-238, *in* Tested studies for laboratory teaching, Volume 15 (C. A. Goldman, Editor). Proceedings of the 15th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 390 pages.

Contents

Introduction

Although the study of evolution is concerned with investigations of *both* ecology and genetics, the popular view of evolution through natural selection dwells primarily on the ecological struggle of organisms with their environment. Genetic factors are, however, just as integral to the process and can greatly influence the course of evolutionary change. For example, the amount and distribution of additive genetic variation and the genetic correlations among traits can play significant roles in determining what can and can not evolve.

Unfortunately, quantitative genetics is a difficult area in undergraduate education. It presents logistic difficulties for the practical laboratory as well as conceptual difficulty for introductory biology students. The phenomenon of inbreeding depression is a notable exception to this generalization. In addition to being virtually a household word in quantitative genetics, its significance and underlying cause can be readily appreciated by undergraduate students, and it is usually powerful enough to be detected by classic experimental analysis. Inbreeding depression is also thought to be a major evolutionary force regulating one of the most prominent evolutionary trends in the flowering plants — the evolution of self-fertilization.

This laboratory exercise involves a term-long experimental study of inbreeding depression in an obligately outbreeding plant, *Brassica rapa*. The exercise requires a full academic term (about 10 weeks) plus 2 weeks of pre-term preparation.

Materials

(for each pair of students)

Planting of Parental Stock (about 2 weeks in advance)

Pots with *four* fertilizer pellets per pot (8)
Pre-moistened germination-grade soil mixture
Watering container
Mat material (30 cm × 25 cm)
Large elastic band (1)

Part A: Experimental Pollinations (the students' first lab)

Brassica rapa, each with open flowers and several buds (8)
Fine forceps, scissors, dissecting needles (2 each)
Masking tape and waterproof markers
Salt solution (15 g NaCl/liter)
Wooden sticks (8)
Spools of thread (2)
Large elastic band (1)

Part B: Harvesting and Planting the Offspring (6 weeks later)

Fine forceps (2)
Pots with *three* fertilizer pellets per pot (8)
Pre-moistened germination-grade soil mixture
Mat material (30 × 25 cm) cut into wicks and watering mats
Large elastic band (1)

Part C: Estimating Fitness and Inbreeding Depression (another 4 weeks later)

Ruler, at least 30 cm in length (2)
Fine scissors (2)
Electronic balance(s) (that weigh to 0.01 g)

Student Outline

Introduction

Inbreeding depression can be defined as *the reduction in fitness of offspring derived from mating between relatives (inbreeding) compared to offspring resulting from mating among unrelated individuals (outcrossing)*. The harmful effects of close inbreeding were widely recognized well before any formal scientific investigation into the phenomenon. Indeed, in humans about 42% of offspring from sister-brother marriages die before they reach reproductive age, hence most, though not all, cultures have strong traditions with respect to incest. Plant and animal breeders have also known for centuries of the superior vigor and yield associated with outbreeding compared to inbreeding. The importance of inbreeding depression in evolutionary biology was established in 1876 with the publication of a book entitled *The Effects of Cross and Self Fertilization in the Vegetable Kingdom* by Charles Darwin. His extensive experiments involving 57 species of plants indicated that inbreeding depression is a widespread and significant evolutionary force.

The most likely cause for the reduction of fitness upon inbreeding involves the expression of *deleterious recessive alleles*. *Recessive* alleles are expressed in homozygotes but remain unexpressed when they occur with a *dominant* allele in heterozygotes. *Deleterious* alleles originate when the underlying DNA sequence of a functional allele is altered by mutation to code for a gene product which is either harmful or simply doesn't work. Since mutation is a universal feature of DNA, all plant or animal populations contain deleterious recessive alleles. At any given locus, however, deleterious alleles are usually so rare that offspring produced through matings among unrelated individuals are almost never homozygous for harmful alleles. With inbreeding the odds of

producing an offspring homozygous for a deleterious allele are much higher. Because rare deleterious mutations are transmitted along family lines, brothers and sisters are much more likely to carrying the same deleterious alleles than unrelated individuals.

In plants, very close inbreeding is possible. More than 70% of flowering plant species are hermaphrodite (i.e., they possess both female and male sex organs), so that it is possible for an individual to mate with itself. This is known as *self-fertilization*. Many plant species regularly self-fertilize, and from a genetic viewpoint this is not surprising. Consider a population of outcrossing hermaphrodites in which a self-fertilizing mutant arises. Outcrossing individuals transmit, on average, one copy of their genes by being a mother to their own seeds and another copy by being a father to some other individual's seeds. The selfing mutant, on the other hand, will be both mother *and* father to its own seeds as well as a father to another individual's seeds. Hence the selfing mutant transmits three copies of its genes to the next generation while outcrossers transmit only two (Table 12.1). This is the *automatic transmission advantage* of self-fertilization.

Table 12.1. The automatic transmission advantage of self-fertilization.

	Gene copies transmitted through:		
	Own seeds	Seeds of others	Total
No Inbreeding Depression			
Outcrossing individual	1	1	2
Selfing individual	2	1	3
With Inbreeding Depression			
Outcrossing individual	1	1	2
Selfing individual	2 (ω_S/ω_O)	1	< 3

Note: The fitness of outcrossed and selfed offspring are ω_O and ω_S, respectively. With inbreeding depression, $\omega_O > \omega_S$.

Although many plants self-fertilize, the majority possess morphological and physiological mechanisms which reduce the chance of self-fertilization and hence promote outcrossing. For example, male and female sex organs are often separated within a flower (known as *herkogamy*) or function at different times during the life of a flower (*dichogamy*). In species such as your study organism (*Brassica rapa*), self-fertilization is prevented by a physiological mechanism known as *self-incompatibility* through which pollen is incapable of functioning on stigmas of the individual from which it came. The question is: Given the automatic transmission advantage of selfing, what selective forces promote the evolution of these outbreeding mechanisms? Or, *what is the evolutionary advantage of outbreeding*? Obviously, inbreeding depression may play a major role here. If the viability of offspring produced through selfing is reduced by inbreeding depression, the transmission advantage of selfing is eroded (see the lower half of Table 12.1). If selfed offspring are only half as vigorous as their outcrossed counterparts, there is no net advantage of self-fertilization.

Measuring Inbreeding Depression

Plants can be ideal study organisms for measuring inbreeding depression. It is relatively easy to regulate who mates with whom, and thereby produce both selfed and outcrossed offspring through experimental hand-pollinations. In addition, the relative fitness of selfed and outcrossed offspring can be compared by growing them in a common environment. Before moving on to the experimental protocol, it is important to become a little more familiar with both the concept of *fitness* in evolutionary biology and your study organism, *Brassica rapa*.

Fitness

Fitness is a central concept in evolutionary biology, and is defined as *the relative ability of an individual to leave descendants and hence transmit its genes to future generations*. This has two components. The ultimate component, sometimes called *fecundity*, is the production of viable offspring which, in turn, go on to produce their own offspring. However, before an individual can produce offspring it must survive to sexual maturity. *Survival* is the other component of fitness. Throughout an organism's life, the probability of dying may differ from one *life-history stage* to the next. Biologists trying to measure survival in plants often break down the life span of a plant into four stages: fertilized ovule to seed; seed to seedling (germination); seedling to adult; and reproductive period as an adult (see Figure 12.1). This approach may, however, prove difficult in long-lived organisms such as trees. Fortunately, your study organism (*Brassica rapa*) is easily grown in the laboratory and completes its entire life-cycle in less than 30 days. You can, therefore, estimate survival for all life-history stages.

Returning to fecundity, the number of viable offspring produced is often difficult to measure. Imagine trying to count and determine the viability of all the maple keys produced on even a single maple tree each year throughout its entire reproductive life. Consequently, biologists often resort to measuring variables which are *correlated with* (i.e., show a close association with) the ability to produce offspring. A biologist working on maple trees may, for example, measure the diameter of the tree trunk as an index of fecundity if there was evidence that trunk diameter (as a measure of tree size) was correlated with maple key production.

In theory, the lifetime fecundity of a short-lived annual plant should be relatively easy to measure. You could grow your experimental offspring in a natural setting where they would be visited by pollinators and produce seeds which, in turn, could be counted and tested for viability. Unfortunately, practical limitations limit your experiment to the laboratory environment. Consequently, you will base your estimates of fecundity on three *fecundity correlates*.

Rapid-Cycling *Brassica rapa*

Brassica rapa is a herbaceous annual mustard in the plant family Brassicaceae (sometimes also called Cruciferae because of their cross-shaped flowers), a plant family which includes many common agricultural species such as broccoli, cauliflower, radish, cabbage, Brussels sprouts, turnips, and rape seed. In natural populations, mating is mediated by an insect pollinator, usually the honeybee, which is lured to flowers by the bright yellow petals and sugary nectar secreted at the base of the flower (Figure 12.2). Wild populations of *B. rapa* are annual, that is they complete their life-cycle in one growing season. The plants you will work with have been selectively bred to complete their life-cycle in an even shorter period of time (Figure 12.3). These “fast plants” or “rapid-cycling” *Brassica*s, as they are often called, are ideal for experiments on inbreeding depression.

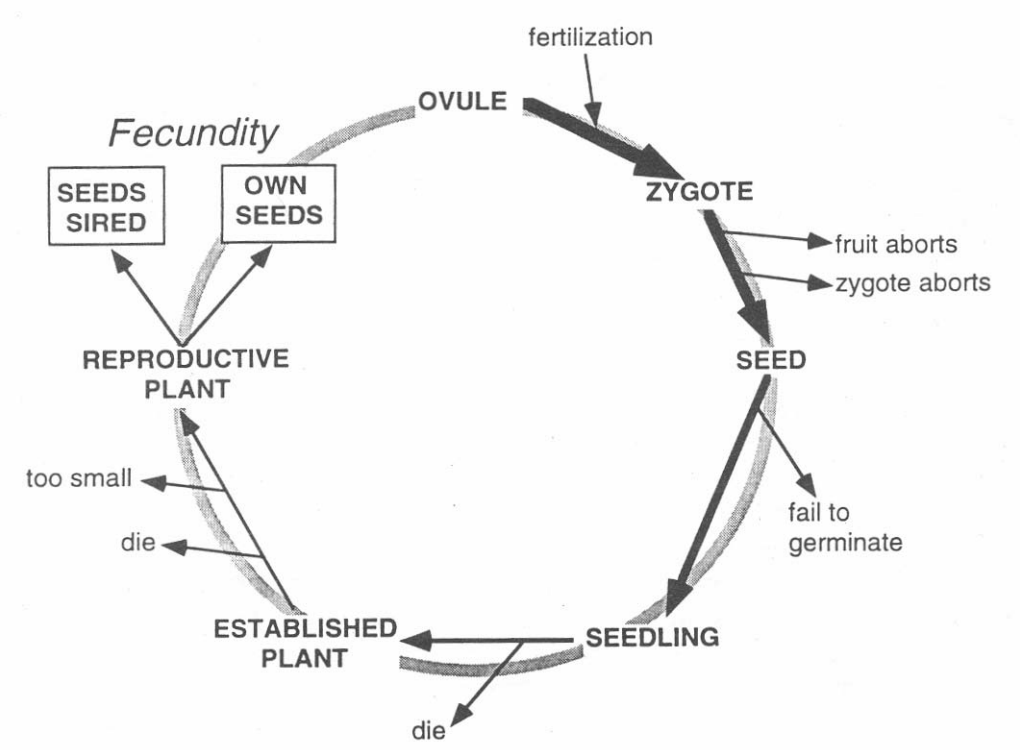

Figure 12.1. Life-cycle hazards and components of fitness in an annual plant.

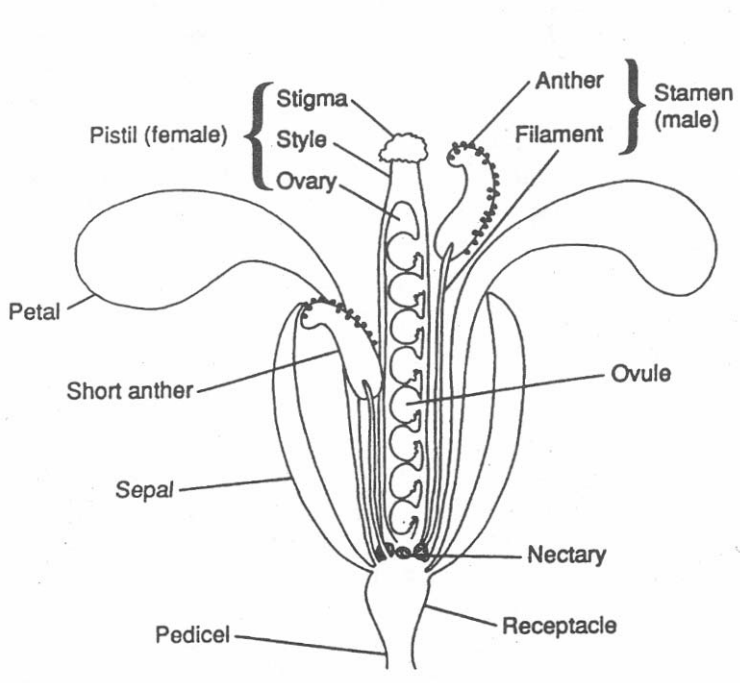

Figure 12.2. Cross-section of a flower of *Brassica rapa*. Reprinted with permission of the Wisconsin Fast Plants™ Program, University of Wisconsin–Madison.

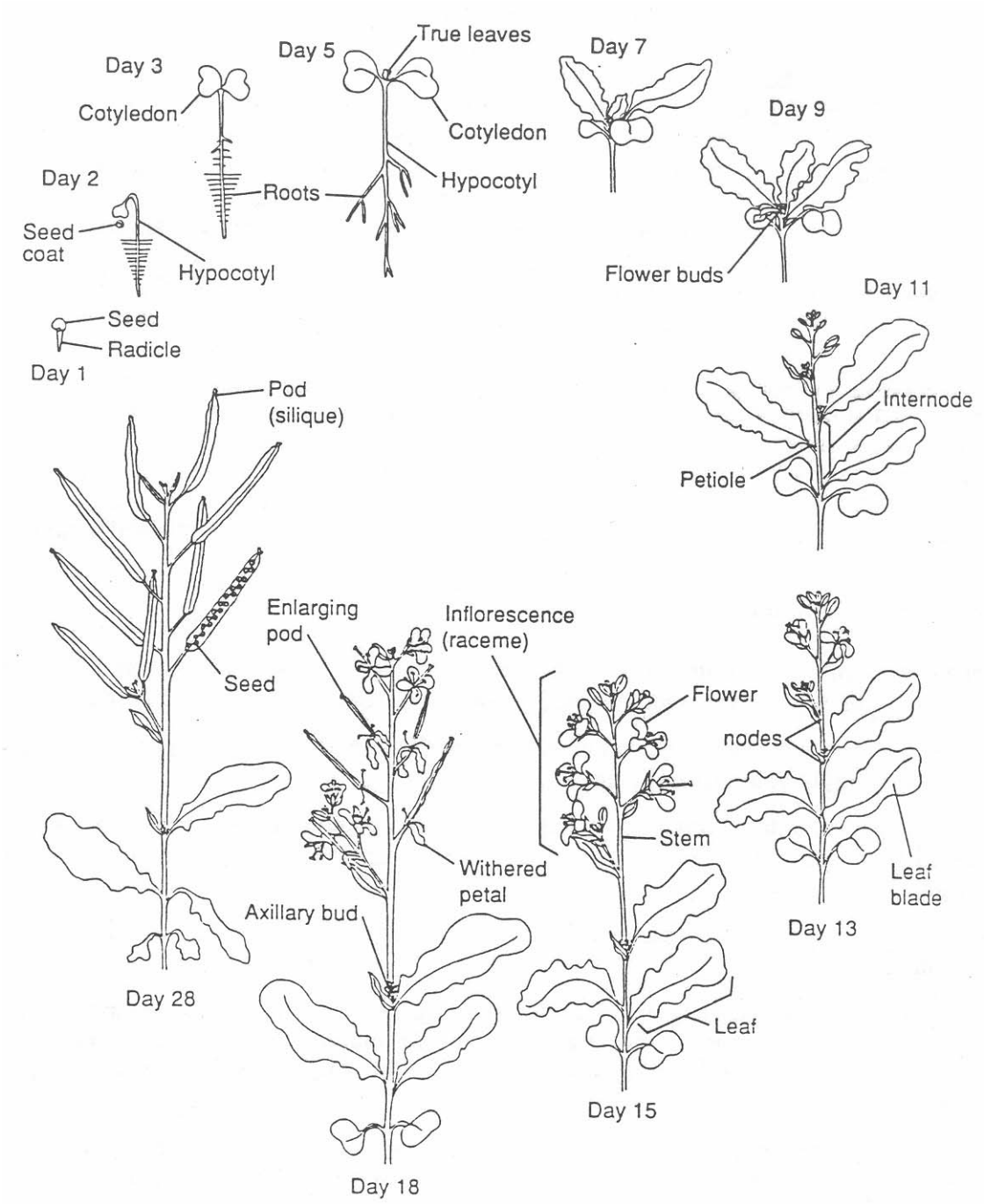

Figure 12.3. The life-cycle of *Brassica rapa*. Reprinted with permission of the Wisconsin Fast Plants™ Program, University of Wisconsin–Madison.

Part A: Experimental Pollinations

In the first part of this study, you will produce seed through both self- and cross-pollination. You will be working with four flowering plants. On two of these, flowers will be self-pollinated; on the other two, flowers will be cross-pollinated. Performing controlled pollinations, whether selfed or outcrossed, simply requires removing pollen from the anthers of the male parent and applying that pollen to the stigma of the female parent. Recall, however, that *B. rapa* possesses a physiological self-incompatibility mechanism which usually prevents successful self-fertilization (see Introduction). To produce selfed offspring you will have to circumvent self-incompatibility.

In *B. rapa*, there are two ways to overcome self-incompatibility. The first method exploits the fact that stigmas are able to able to receive pollen about 3 days before the flowers open, yet the self-incompatibility system only develops about 2 days before the flowers open. This affords 1 day of bud development during which flowers can be self-fertilized. Finding buds at the right stage is relatively easy since flowers open sequentially every 8 hours. However, it is difficult to pollinate young buds since they are very small and delicate. An alternative way of disrupting self-incompatibility is to treat the stigma surface with salt just before pollination. The incompatibility reaction in *B. rapa* occurs on the surface of the stigma, hence altering the stigmatic environment by the application of a weak salt solution abolishes the incompatibility reaction and allows successful self-fertilization.

I – Performing Pollinations

1. With masking tape and a waterproof marker, label each pot with your initials and indicate whether the plant will be selfed (S) or outcrossed (O). You will use two plants per pollination treatment.
2. Find the most recently opened flower and locate the first three buds up the stem (the oldest buds). These are the buds you will pollinate.
3. Using forceps and a dissecting needle, carefully open the bud to expose the stigma as illustrated in Figure 12.4 (A and B). Do not rip off the sepals and petals; simply part them to fully expose the stigma.
4. Gently remove the anthers. Note that they are not yet mature and should not be releasing pollen at this stage.
5. Apply a drop of salt solution to the three stigmas using a wooden stick. Spread the solution around to fully cover the surface of the stigma. Wait *15 minutes* for the salt to breakdown the incompatibility system, and then dry off the stigma with a tissue to remove any remaining salt solution.
6. Using forceps, gently remove a mature anther from a recently opened flower on one of your *other* plants (for outcrossing) or the *same* plant (for selfing) and rub the pollen-covered surface of the anther lightly across the exposed stigmas of the three buds (Figure 12.4C), making sure some pollen adheres to the stigma surfaces.
7. Gently tie a small piece of thread around the base of each pollinated bud to help identify it in the next lab.
8. Remove all *unpollinated* buds (i.e., buds 4, 5, 6, etc.). Nip off the apical meristem and remove all other inflorescences and axillary buds (see Figure 12.3) but leave a couple of recently opened flowers for a source of outcross pollen. Do *not* remove any leaves.

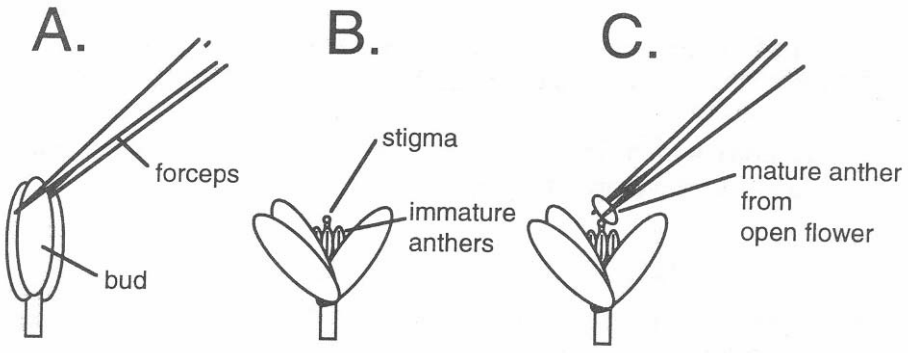

Figure 12.4. Bud pollination in *Brassica rapa*. Fine forceps are used to carefully expose the stigma (A and B) and lightly brush a mature anther across the stigma surface (C). Note that the anthers in the bud are not mature. Pollen must be obtained from mature anthers on recently opened flowers.

9. Once all of your plants have been pollinated, remove all of the open flowers on each plant, leaving only the buds you pollinated. With the flowers and unpollinated buds removed, the plant can devote more resources towards maturing the flowers you pollinated.
10. To help support the plant during fruit maturation, attach stakes to each using the thread and wooden sticks provided.
11. Combine plants with your neighbor and place all eight, with their pots held together lightly by an elastic band, back on the watering container.
12. Place the watering containers back under the lights on the growth bench. The selfed and outcrossed seeds will be mature in 6 weeks.

II – Questions

1. In the Introduction, you learned that deleterious alleles are almost always *recessive*. Why are *dominant* deleterious alleles (i.e., those which cause harmful effects even in the heterozygous condition) not maintained in populations while deleterious recessives are?
2. Do you think that *B. rapa* normally self-fertilizes in natural populations? Explain. Consider the features of floral morphology which may indicate whether this species normally selfs or outcrosses.
3. Why did you use buds for *both* self- and outcross-pollinations? Why did you not use open flowers for cross-pollination instead?

Part B: Harvesting and Planting the Offspring

In this part of the experiment, you harvest and count the pods and seeds produced by your self- and cross-pollinations. You will then take a sample of seeds and plant them in a growth experiment to compare the relative fitness of selfed and outcrossed progeny.

I – Fruit and Seed Collection

In the previous part of the experiment you pollinated *three* buds on each of *four* plants; two of these plants were selfed and two were outcrossed.

1. Remove the mature pods which developed from the buds you pollinated, and record in Table 12.2 those which failed to develop into pods.

Table 12.2. Pod and seed production.

	Buds pollinated	Pods matured	Pods per pollination	Total seeds	Seeds per pod
Outcrossed					
Plant 1					
Plant 2					
Selfed					
Plant 1					
Plant 2					

2. For each plant, carefully split open each pod and count the number of seeds inside. Mature seeds should be brownish and plump. Smaller, shriveled seed-like things are aborted ovules and should *not* be counted as seeds. Record the data in Table 12.2 and then add your results to the class data.
3. Remove the plants from their pots and discard them. Wash out the pots and watering container, and discard the used watering mat.
4. Record the class data in Table 12.3 and perform Student's *t*-tests to evaluate whether "pods per pollination" and "seeds per pod" differ significantly between cross- and self-pollinations.

II – Planting Selfed and Outcrossed Offspring

Your goal is to compare the survival and growth of the selfed and outcrossed offspring under *uniform* conditions. Although growing conditions will always vary to some extent from pot to pot, you should do everything possible to minimize this environmental variation.

1. Set up *four* pots as follows:
   (a) Using forceps, pull the thin end of one water wick through the bottom of each pot until half the wick is sticking out of the bottom (see Figure 12.5).
   (b) Lightly place some soil in the pot and tap it down by rapping the bottom of the pot on the lab bench. Do not use your finger to jam soil into the pot. *Do not pack soil too tightly.*

Table 12.3. Statistical analysis of pod and seed production.

	Outcrossed	Selfed	Results of *t*-test
Pods/pollination			*t*-value = _____ *df* = _____ _____ < *P* < _____ Accept or reject H_0?
Mean			
Standard deviation			
Sample size			
Seeds/pod			*t*-value = _____ *df* = _____ _____ < *P* < _____ Accept or reject H_0?
Mean			
Standard deviation			
Sample size			

(c) When the pot is *half* full, place *three* fertilizer pellets in the pot and cover with soil until the pot is almost full. Since the soil you are using is very low in nutrients, fertilizer is critical to plant nutrition. These pellets slowly release nitrogen, phosphorus, and potassium over the course of the experiment.

2. You will plant two outcrossed and two selfed seeds (one per pot). Try to use one seed from each of your maternal plants. If one or more plants produced no seed, substitute another seed from the appropriate treatment (i.e., selfed or outcrossed).

3. Each seed should be planted by grasping it gently with forceps and pushing it into the soil in the center of the pot to a depth of *only 1 or 2 mm.* If you've done all this properly, each of your pots should be set up as illustrated in Figure 12.5.

4. With your neighbor, set up a watering container, place your eight pots on the watering mat (*alternating selfed and outcrossed* so that selfed or outcrossed offspring are not clustered together), and bind the pots together gently with an elastic band. Ensure that the wicks are in good contact with the watering mat.

5. Set up the watering container under the lights on the growth bench. They will be ready to measure in 4 weeks.

III – Questions

1. What are the probabilities that the average differences in pods/pollination and seeds/pod between selfed and outcrossed offspring (Table 12.3) are due to random chance rather than inbreeding depression?

2. Why was it important to *not* have offspring from one pollination treatment clustered together on the growth bench?

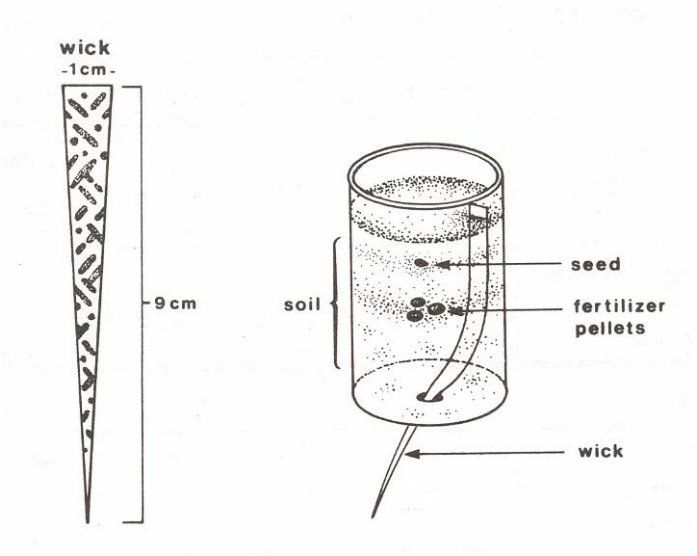

Figure 12.5. Proper pot set-up.

Part C: Estimating Fitness and Inbreeding Depression

In this part of the experiment, you will compare the relative survival and fecundity of selfed and outcrossed offspring grown in a common environment. First, you will calculate survival from seed to seedling and from seedling to adult. Then, you will measure three characteristics thought to be correlated with fecundity. Finally, you will synthesize the data from the entire experiment, perform the appropriate statistical analyses, and draw conclusions on the strength and evolutionary consequences of inbreeding depression in *B. rapa*.

I – Seed and Seedling Survival

1. Retrieve your four pots and closely inspect each to determine whether the seed germinated. Germination is indicated by emergence of the root or shoot from the seed. Note also which of the germinated seeds survived to produce adult plants. Enter the germination and survival data in Tables 12.4 and 12.5, respectively.
2. Add your data to the class data and once the totals have been tallied perform a chi-squared (X^2) test to evaluate whether germination and survival differs significantly between selfed and outcrossed offspring.

Table 12.4. Germination.

	Number of seeds:		Proportion germinated
	Germinated	Not germinated	
Your data			
Outcrossed			
Selfed			
Class data			
Outcrossed			
Selfed			
X^2 = _____; *df* = ____; _____ < *P* < _____; Accept or reject H_0?			

Table 12.5. Survival.

	Number of seedlings:		Proportion survived
	Alive	Dead	
Your data			
Outcrossed			
Selfed			
Class data			
Outcrossed			
Selfed			
X^2 = _____; *df* = ____; _____ < *P* < _____; Accept or reject H_0?			

II – Measurement of Fecundity Correlates

1. You will measure three characteristics of your plants that may be correlated with fecundity (the ability to produce viable seeds). Enter your data in Table 12.6 and add your numbers to the class data.
   (a) First measure *plant height* (to 0.1 cm) from the soil surface to the highest apical meristem.
   (b) Next, count the *number of flowers*; both those that are open and previously opened flowers that are now withered.

(c) Finally, cut the stem at soil level and measure the *fresh weight* of the plant (to 0.01 g) using one of the electronic balances provided. Plants that have prematurely dried out should *not* be weighed but can provide data on both flower number and plant height.

Table 12.6. Fecundity correlates.

	Plant height	Number of flowers	Fresh weight
Outcrossed			
Plant 1			
Plant 2			
Selfed			
Plant 1			
Plant 2			

2. Remove the plants from their pots and discard them. Wash out the pots and the watering container and discard the used watering mat.
3. Record the class data in Table 12.7 and perform Student's *t*-tests to evaluate whether the three fecundity correlates differ significantly between selfed and outcrossed offspring.

III – Data Analysis

Differences between selfed and outcrossed offspring are conventionally summarized by the parameter δ (delta) which represents the strength of inbreeding depression, and is calculated as:

$$\delta = 1 - \left(\frac{\omega_S}{\omega_O} \right)$$

where ω_S and ω_O are the fitnesses of selfed and outcrossed offspring, respectively. Note that δ increases with the strength of inbreeding depression. Positive values mean that outcrossed offspring have a higher fitness than selfed offspring; negative values mean just the opposite. When $\delta = 0$ inbreeding has no effect on offspring fitness. You may calculate δ separately for survival during each of the three life-history stages, and for each fecundity correlate by substituting the mean of selfed offspring for ω_S and the mean of outcrossed offspring for ω_O.

An overall estimate of inbreeding depression that makes use of all the data you have collected can be derived by calculating a *multiplicative fitness function* (W) for both selfed (W_S) and outcrossed (W_O) offspring and then calculating:

$$\delta_t = 1 - \left(\frac{W_S}{W_O} \right)$$

Table 12.7. Statistical analysis of fecundity correlates.

	Outcrossed	Selfed	Results of *t*-test
Plant height			*t*-value = _____ *df* = _____ _____ < *P* < _____ Accept or reject H_0?
Mean			
Standard deviation			
Sample size			
Number of flowers			*t*-value = _____ *df* = _____ _____ < *P* < _____ Accept or reject H_0?
Mean			
Standard deviation			
Sample size			
Fresh weight			*t*-value = _____ *df* = _____ _____ < *P* < _____ Accept or reject H_0?
Mean			
Standard deviation			
Sample size			

The multiplicative fitness function combines the probabilities of survival at each of the life-history

$$W = \left(\frac{pods}{pollination} \right) x \left(\frac{seeds}{pod} \right) x \left(germination \right) x \left(survival \right) x \left(fecundity \right)$$

stages with your measure of fecundity:
Since you have not measured fecundity directly, correlates of fecundity will be substituted in its place. Complete Table 12.8 by calculating δ for all the parameters measured, and δ_t using each of the three fecundity correlates. Calculate the average of the three δ_t's. This is probably your best overall measure of inbreeding depression in *B. rapa*.

IV – Questions

1. Are selfed offspring *generally* less fit than outcrossed offspring? Which components of fitness show statistically significant inbreeding depression?
2. For which aspect of offspring viability is inbreeding depression the strongest? For which is it weakest?
3. Which of the three fecundity correlates probably best predicts actual fecundity in *B. rapa*? Explain.

Table 12.8. Estimates of inbreeding depression in *Brassica rapa*.

	P/P	S/P	%G	%S	PH	NF	FW	W_{PH}	W_{NF}	W_{FW}
Outcrossed										
Selfed										
ω_S/ω_O										
δ (or δ_t)										
Average of the three δ_t's = _____										

Note: P/P = pods/pollination; S/P = seeds/pod; %G = proportion of the seeds germinated; %S = proportion of seedlings that survived; PH, NF, and FW = plant height, number of flowers, and fresh weight, respectively. Each of the *W*s is the multiplicative fitness based on the subscripted fecundity correlate.

4. Do you think self-fertilization is likely to evolve in *B. rapa*? Explain in terms of the theory concerning the evolution of self-fertilization (i.e., calculate the number of gene copies transmitted by outcrossing and selfing individuals as in Table 12.1).

Notes for the Instructor

This lab exercise is based on a straightforward experiment. There are, however, a few useful tips for potential instructors. The section below gives details on culture conditions and equipment as well as specific procedural tips. Results from a pilot study and references for background information are provided in Appendices A and B, respectively.

Culture Conditions and Equipment

The most critical factor in the success of this exercise is the health and growth of the plants. Below, are the most important features of the growth environment that was used successfully at the University of Toronto. Obviously there is more than one way to grow Fast Plants, and experience will suggest alternatives. This particular method, however, can be used as a starting point. Much useful information is also provided in a publication by Stephen Tomkins and Paul Williams (*Journal of Biological Education*, 24: 239–250, 1990) and in the *Wisconsin Fast Plants™ Manual* sold by Carolina Biological Supply (800-334-5551, #15-8950, $16 US).

Pots, Soil, and Fertilizer

Plants are grown singly in small, independent pots (see Figure 12.5). It is important that pots can be separated, since plants must be able to be shuffled around according to stage of flowering and fruiting. For this reason, the "quads" (four-celled styrofoam units) typically used to grow fast plants are unsuitable. Individual pots can be made from plastic test tube covers, with holes drilled in the top (bottom) for wick placement, but anything similar will do. The potting medium should be a fine-grade (germination grade) soil-less mix (Carolina sells a special Fast Plant soil, #15-8966, $36 US per 2 cu ft, enough for about 4000 pots). Nutrients are supplied by slow-release fertilizer pellets (Carolina, #15-8971, $9 US per lb, three pellets per pot).

Lighting and Temperature

It is probably best to grow all the plants involved in this experiment in the same environment. Fast Plants have been bred to grow well under fluorescent lighting at room temperature. So, growth benches can be set up at the back of a regular lab room. Lighting should be supplied by cool white bulbs spaced about 10 cm apart and kept 5–10 cm away from the plants at all times (i.e., the height of the light bank must be adjustable). Especially fast-growing plants should be monitored regularly so that they do not grow up into the hot light tubes. Carolina sells a portable lighting stand for Fast Plants (#15-8998, $112 US) as well as several other mobile plant growth stands.

Watering

Individual pots are kept watered via their wicks (see Figure 12.5) which are in contact with a moist watering mat. The wicks and mats can be made from any stable absorbent material such as Kimtuff™ Wipe, heavy duty J-cloths, or the fabric "pellon" (shoulder stuffing). *Note that all mat and wick material should be washed with detergent and rinsed thoroughly before use.* Inexpensive watering containers may be made from 500-ml yogurt containers (Figure 12.6). These are then set in green plastic trays (e.g., Carolina #66-5918, $6 US each). The water flows freely among the containers set in each tray, hence, adding water and inspecting water quality is easy. To inhibit the growth of algae on the mats and wicks, copper sulfate should be added directly to the water supply. One of Carolina's Anti-Algal Squares (#15-8979, $1 US each) per watering container should do the trick.

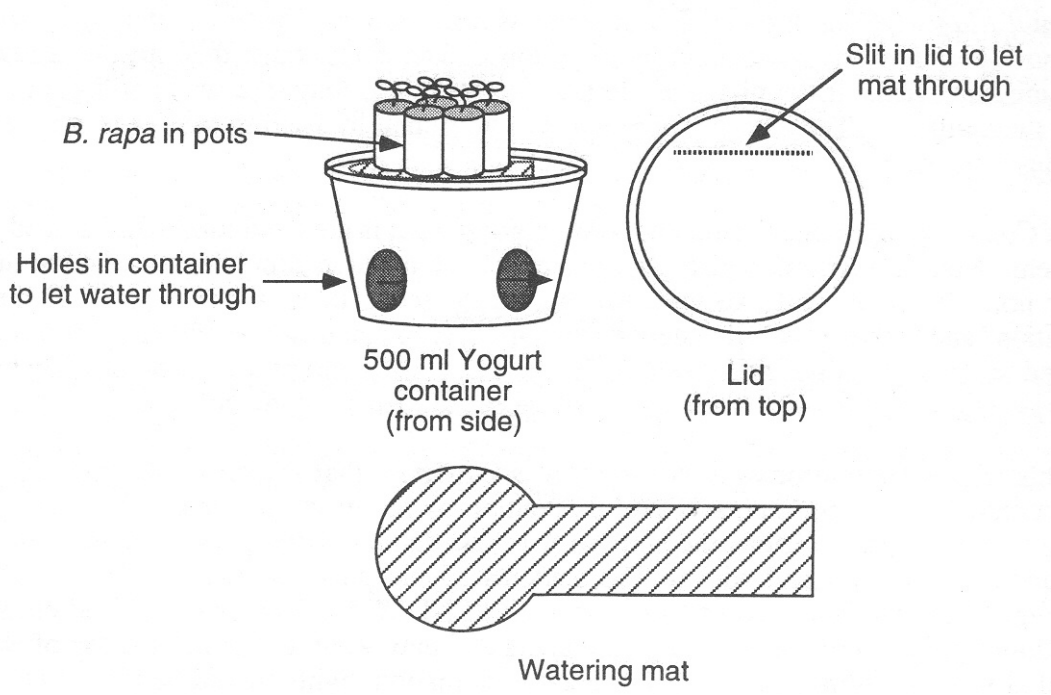

Figure 12.6. An inexpensive watering system for fast-cycling *Brassica rapa*.

Parental Stock

The parental plants are basic fast plant stock distributed by Carolina (#15-8811, $16 US for 200 seeds). They should be planted about 2 weeks in advance of the first lab period. Heat waves or cools spells can alter the growth schedule so that it is best to hedge your bets by staggering planting, rather than doing it all on one day. *On the first lab day parental plants must possess at least one or two open flowers plus at least three well-developed buds*. It is best to "calibrate" the timing of the Fast Plant life-cycle to your particular growth conditions by cultivating some plants *in situ* before you attempt the lab on a large scale

Procedural Tips

Part A: Experimental Pollinations

Undergraduate students often have little opportunity to work directly with plants, so they may require close attention while doing experimental pollinations. Obviously, this is a critical step. If the pollinations don't work, the experiment can't be continued. Here are some things to watch out for: (a) sepals and petals need only be moved a little bit to expose stigmas; (b) dry off excess salt solution *before* pollinating; (c) exert vigilant quality control over the plants that are returned by the students, and weed out anything suspicious; and (d) get the students to pollinate as many plants as they have time for — the more pollinations, the greater the chance of having enough seeds to complete the experiment with adequate sample sizes.

Part B: Harvesting and Planting the Offspring

Taking Parental Plants Off the Water: Parental plants *must* be taken off the water right when the pods are mature (i.e., they are *just* beginning to yellow). The plants must then dry for a few days before seeds are harvested. If the plants are left on the water too long, the seeds will germinate in the pods and they will die. The entire experiment can be ruined by leaving the plants on the water a couple of days too long.

Pod and Seed Counting: Students should know what is and what is not a mature *Brassica* seed before they count them. Note that *Brassica* pistils spontaneously elongate to some degree whether they are pollinated or not. A small, pod-like structure with zero seeds in it is not a pod. Remember, "pods/pollination" and "seeds/pod" are independent variables so that unsuccessful pollinations should *not* be counted as zero seeds per pod. Instructors should closely monitor the compilation of class data, and suspicious values should be checked before the students record the data for analysis.

Planting: This step is straightforward, however, it is important that students plant the right seeds (selfed or outcrossed) in the right pots. Careful attention by the instructor helps. It also expedites matters if the instructor pre-moistens the soil (so it is *just* damp — *dry, peaty soil will not work*), and washes and cuts the mats and wicks. The students should plant as many seeds as possible to maximize sample sizes for fitness comparisons (see APPENDIX A). Instructors should ensure that pots are positioned properly on the watering containers and may want to "prime" the top of each pot with a few ml of water to initiate wicking. For good germination, lights should be lowered to within 5 cm of the soil surface.

Part C: Estimating Fitness and Inbreeding Depression

It is now about 10 weeks since the experiment began. At this point, it is worthwhile reviewing the theory behind the evolution of plant mating systems. The students should begin this part of the lab with the feeling that they are testing a hypothesis of considerable importance in evolutionary biology.

Germination and Survival: Again, germination and survival are independent parameters, so that only seeds which germinated are included in the survival estimates. This requires that students take care to distinguish non-germinated seed from those dying shortly after germination. Compilation of class data should be closely monitored.

Estimation of Fecundity: As presented above for this lab, the estimation of fecundity is based on three easily measured characteristics: plant height, number of flowers, and fresh weight (fresh weight is highly correlated with dry weight in *B. rapa*). The study, however, could be expanded to include other variables such as measures of leaf area and/or chlorophyll production (photosynthetic capacity), ovule production (female reproductive capacity), or pollen production (male reproductive capacity). Inbreeding effects generally increase with age in *B. rapa*, so that offspring should be allowed to grow for about 4 weeks. Again, tight control on the quality of the data is very important since erroneous data points can have a large effect with small sample sizes. Make sure that students know precisely how to perform these measurements. Data may be pooled between concurrent lab groups to increase statistical power, however, this will require that different groups are doing measurements the same way. If you do pool data, the students should know what is going on, since after 10 weeks they may feel some sense of propriety over their work.

Analysis: In the exercise presented above, it is assumed that the students are familiar with both the chi-squared (X^2) test and Student's *t*-test. If not, the experiment can be a vehicle for introductory statistics or can be done with no formal statistical analysis. It helps if the instructors spend some time defining the final parameters in concrete terms using simple examples (e.g., W_{FW} is the amount of surviving biomass resulting from a single self or cross pollination). It is also worth discussing how the final δ is likely to be an underestimate of the inbreeding depression that would be expressed under harsher, more natural conditions. Encourage the students to consider important parameters that have not been incorporated into the experiment.

Acknowledgments

I thank Spencer Barrett, Mike Dennison, Sean Graham, Grant Hurlburt, Bob Latta, Ali Mobasheri, Chris Permulla, and especially Corey Goldman for helpful suggestions throughout the original development of this exercise; and Corey Goldman and ABLE for the opportunity to share this lab and for their swift and attentive editorial assistance.

APPENDIX A
Results of a Pilot Study

The following are results from the original pilot study for this exercise. The experiment was conducted under the growth conditions described above, and pollinations, planting, and measurements were not performed in an overly-fastidious manner (i.e., the variances should resemble those obtained under teaching-lab conditions).

Table 12.9. Statistical analysis of pod and seed production in the pilot study.

	Outcrossed	Selfed	Results of *t*-test
Pods/pollination			*t*-value = 1.187 *df* = 19 $0.25 < P < 0.50$ Accept H_0
Mean	0.81	0.92	
Standard deviation	0.24	0.15	
Sample size	9	12	
Seeds/pod			*t*-value = 0.357 *df* = 19 $0.75 < P < 0.90$ Accept H_0
Mean	8.83	8.25	
Standard deviation	3.37	3.93	
Sample size	9	12	

There is little effect of inbreeding on pod and seed production. Increasing sample sizes only serves to ensure a good supply of selfed and outcrossed seed. Note, these data indicate that the salt treatment was completely effective in abolishing the incompatibility reaction.

Table 12.10. Germination in the pilot study.

	Number of seeds:		Proportion germinated
	Germinated	Not germinated	
Your data			
Outcrossed	2	0	1.00
Selfed	1	1	0.50
Class data			
Outcrossed	34	6	0.85
Selfed	24	11	0.72
$X^2 = 1.867$; *df* = 1; $0.10 < P < 0.25$; Accept H_0			

Table 12.11. Survival in the pilot study.

	Number of seedlings:		Proportion survived
	Alive	Dead	
Your data			
Outcrossed	2	0	1.00
Selfed	1	0	1.00
Class data			
Outcrossed	26	8	0.76
Selfed	19	10	0.65
$X^2 = 0.920$; $df = 1$; $0.25 < P < 0.50$; Accept H_0			

The results above reveal a non-significant trend towards a reduction of germination and survival of selfed offspring. Further experience has shown that this trend becomes statistically significant with increased sample sizes.

Table 12.12. Statistical analysis of fecundity correlates in the pilot study.

	Outcrossed	Selfed	Results of *t*-test
Plant height			*t*-value = 2.883 *df* = 43 $0.001 < P < 0.01$ Reject H_0
Mean	28.37	16.51	
Standard deviation	15.09	11.29	
Sample size	26	19	
Number of flowers			*t*-value = 2.268 *df* = 39 $0.025 < P < 0.05$ Reject H_0
Mean	46.3	21.1	
Standard deviation	41.0	20.4	
Sample size	25	16	
Fresh weight			*t*-value = 2.146 *df* = 43 $0.025 < P < 0.05$ Reject H_0
Mean	3.080	1.862	
Standard deviation	2.100	1.495	
Sample size	26	19	

The harmful effects of inbreeding compound throughout the life-cycle, producing significant differences between small samples of selfed and outcrossed offspring. Note that number of flowers was calculated only from those offspring which reached flowering. It is equally reasonable to include non-flowering offspring as zeros.

Table 12.13. Estimates of inbreeding depression in *Brassica rapa* in the pilot study.

	P/P	S/P	%G	%S	PH	NF	FW	W_{PH}	W_{NF}	W_{FW}
Outcrossed	0.81	8.83	0.85	0.76	28.4	46.3	3.08	131	214	14.2
Selfed	0.92	8.25	0.72	0.65	16.5	21.2	1.86	59	75	6.6
ω_S/ω_O	1.13	0.93	0.85	0.85	0.58	0.46	0.60	0.45	0.35	0.46
δ (or δ_t)	-0.13	0.07	0.15	0.15	0.42	0.54	0.40	0.55	0.65	0.54
Average of the three δ_ts = 0.58										

Note: P/P = pods/pollination; S/P = seeds/pod; %G = proportion of the seeds germinated; %S = proportion of seedlings that survived; PH, NF, and FW = plant height, number of flowers, and fresh weight, respectively. Each of the *W*s is the multiplicative fitness based on the subscripted fecundity correlate.

The results of the pilot study indicate that, even under benign environmental conditions, inbreeding depression is strong enough to prevent the spread of a self-fertilizing mutant in *B. rapa*. That is, an outcrosser passes on 2.00 copies of it genes compared to 1.84 copies transmitted by a fully selfing mutant.

APPENDIX B
Sources of Background Information

Here are some references for background information on inbreeding depression as well as experimental studies of the significance of inbreeding depression in the evolution of plant mating systems. These experimental studies could be used to expand the intellectual scope of the experiment to upper undergraduate levels.

Background Reading

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