Appendix A
Effect of Population Density and Mechanism of Sexual Development in Two Strains of C-Fern Gametophytes

Introduction

In this exercise we will look at gametophytes of the tropical fern *Ceratopteris richardii* (C-Fern). C-Fern is a model system for studying plant growth and development since it has a very rapid life cycle and is easy to grow *in vitro*. Ferns are vascular seedless plants. As is true of all plants, their life cycle (Figure 1) involves an alternation between a multicellular diploid generation (**sporophyte**) and a multicellular haploid generation (**gametophyte**). In ferns the gametophytes and sporophytes exist as separate, nutritionally independent forms. The sporophyte grows much larger (up to several meters in the case of tree ferns) than the gametophyte and is the form that we are used to seeing. Sporophytes undergo meiosis in their sporangia to produce haploid **spores** (Figure 2). In the presence of light and moisture these spores germinate within 3-4 days and undergo mitosis to produce the gametophyte (Figures 2 and 3). These gametophytes form **rhizoids**, root-like structures that are responsible for absorbing water and minerals, and a **prothallus**, a leaf-like structure that carries out photosynthesis.

At 28°C and high humidity, the gametophytes of C-Fern reach sexual maturity in 10-12 days after plating the spores. The first spores to germinate become hermaphrodites, which are mitten-shaped, and form the specialized gamete producing structures known as **archegonia** that produce a single egg, and **antheridia** that produce 16 sperm each (Figure 4). As the hermaphrodites develop, they produce a pheromone, **antheridiogen**, which causes later germinating spores to become male. Male gametophytes are tongue-shaped (Figure 5) and produce a large number of antheridia that are quite obvious (they look like pimples). Hermaphrodites possess a **meristem**, a region of rapidly dividing cells, which allows them to grow much larger than the ameristic males. In a dense population the concentration of antheridiogen is high, resulting in a high proportion of male gametophytes.

In the presence of water the antheridia burst open, releasing the motile sperm (Figure 5). The sperm swim, using their flagella, towards a high concentration of an unknown chemical released from the archegonia (this process, known as **chemotaxis**, can be simulated in the laboratory using malic acid). One sperm will fertilize the egg, resulting in a diploid zygote that undergoes embryogenesis to form the new sporophyte (Figure 6). The new sporophyte literally grows right out of the archegonium.

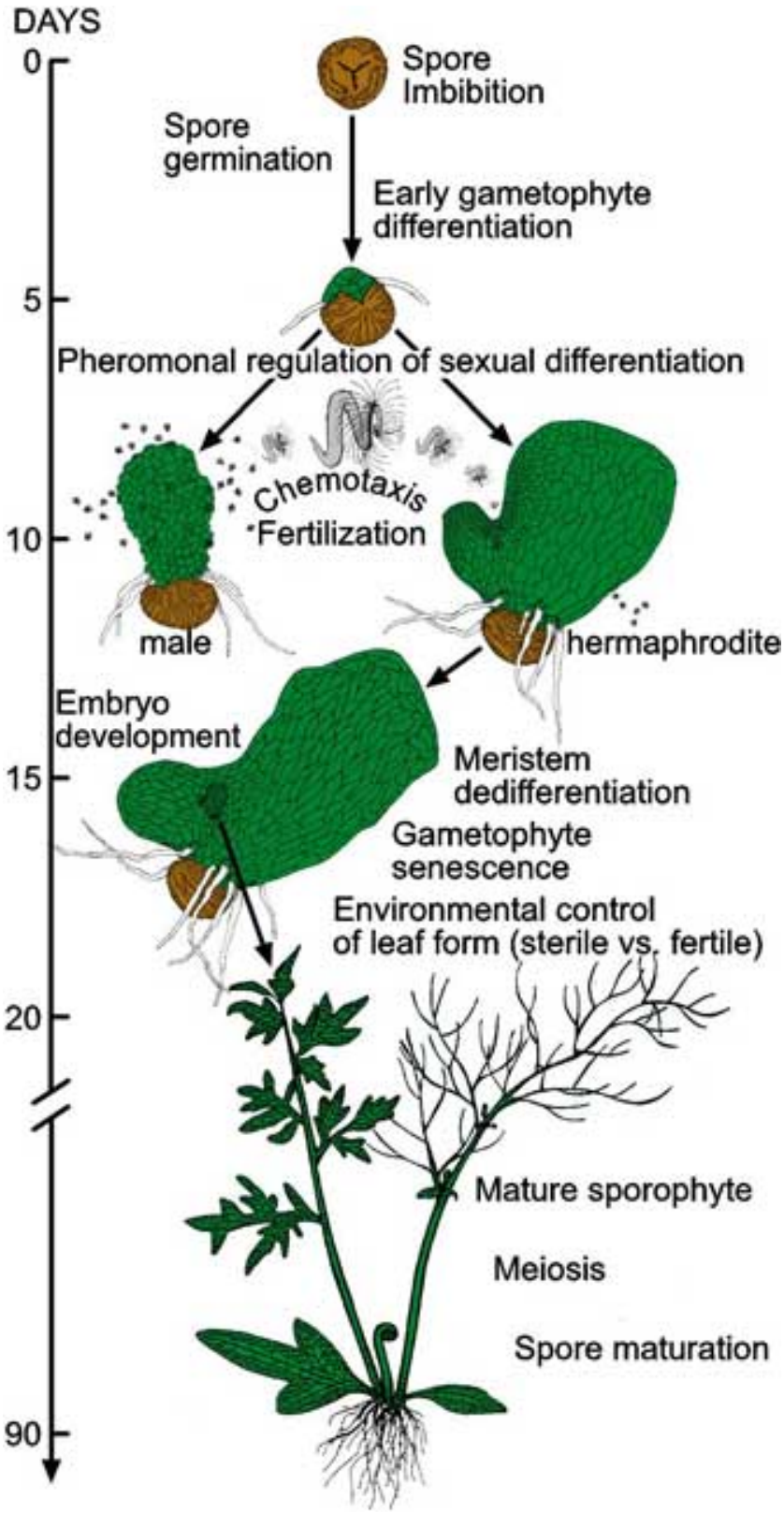
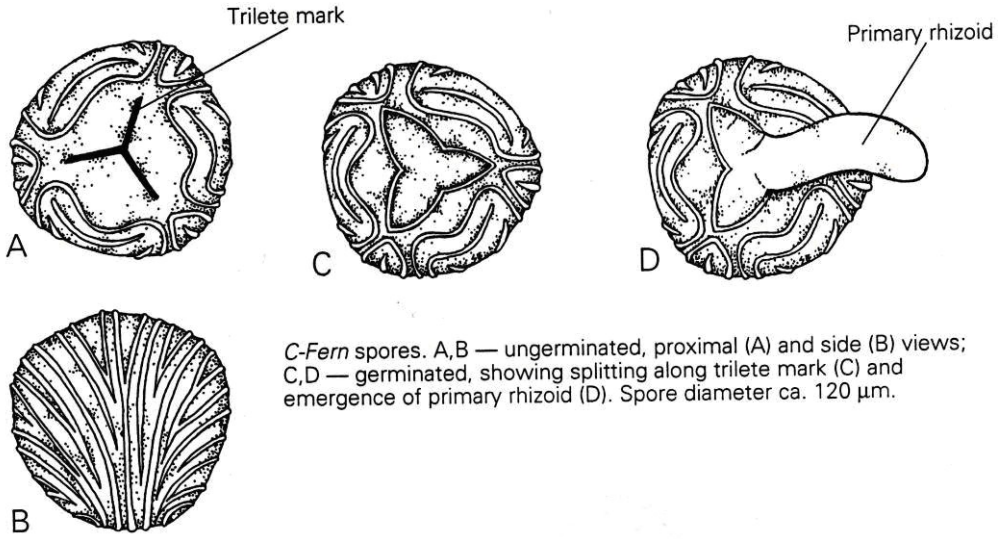


Figure 1. Sequence of developmental events in C-Fern. From (1).



C-Fern spores. A,B — ungerminated, proximal (A) and side (B) views; C,D — germinated, showing splitting along trilete mark (C) and emergence of primary rhizoid (D). Spore diameter ca. 120 μm.

Figure 2. Stages of gametophyte and early sporophyte development. From (1).

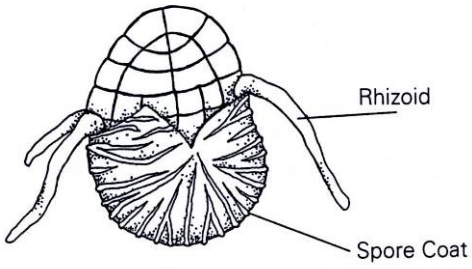


Figure 3. Young C-Fern gametophyte, 5 days from start (DFS) of culture. Spore coat diameter ca. 120 μm. From (1).

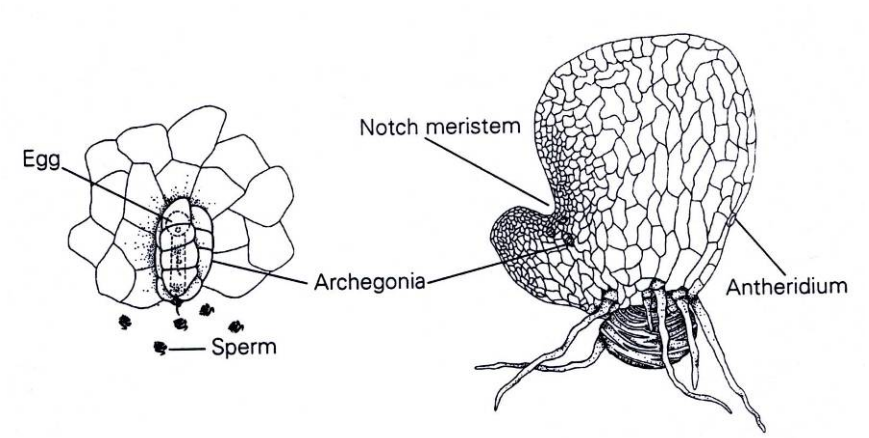


Figure 4. Mature hermaphroditic C-Fern gametophyte with archegonia behind the notch meristem and a single antheridium on the margin, ca. 10 DFS. Spore coat diameter ca. 120 μm. Close-up: view of mature archegonium during fertilization. Sperm enter the open neck canal, uncoil and move toward the egg. From (1).

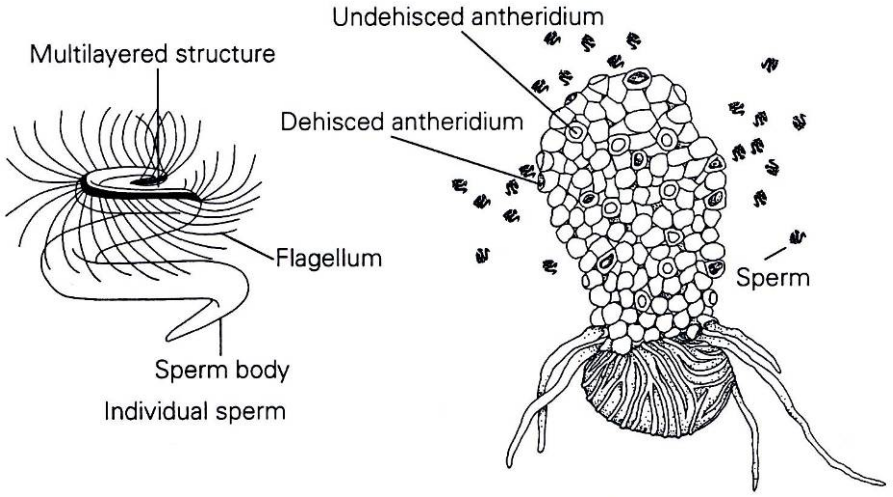


Figure 5. Mature male C-Fern gametophyte, ca.10 DFS. Sperm are released from the numerous antheridia on the surface of the male gametophyte. Spore coat diameter ca. 120 μm. From (1).

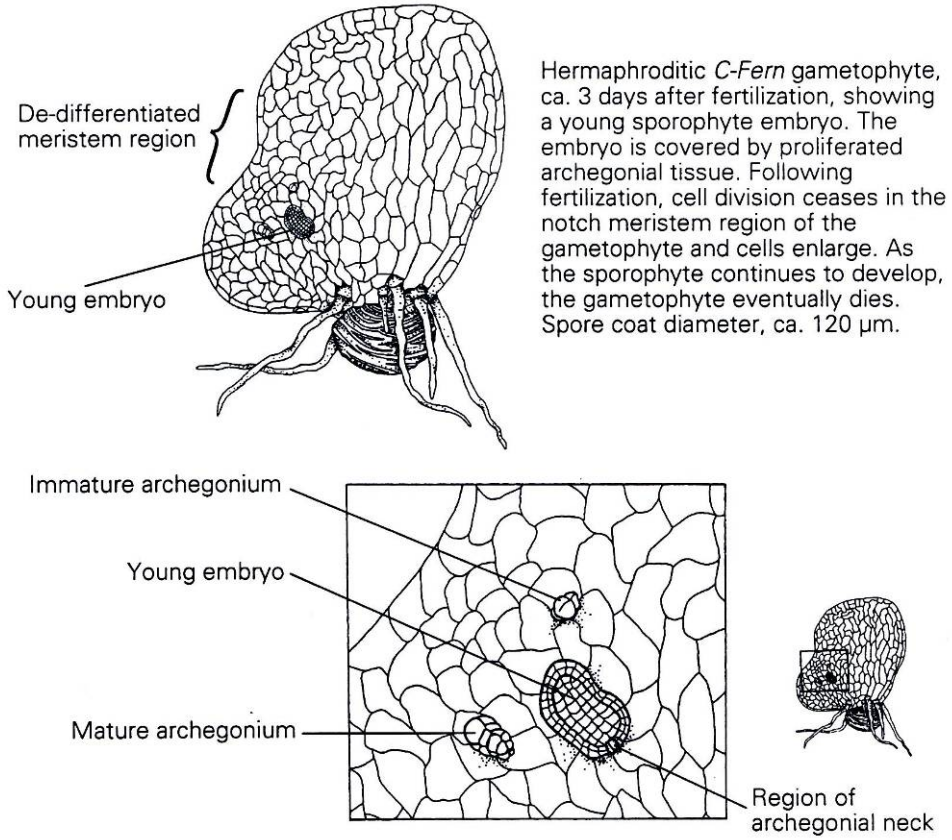


Figure 6. Close-up of hermaphroditic C-Fern gametophyte, ca. 3 days after fertilization, showing unfertilized (immature and mature) archegonia and young embryo developing within proliferated archegonial tissue. The remains of the old archegonial neck can be seen at the distal end of the embryo. From (1).

This lab consists of three parts. First we will look at the effect of population density on the ratio of males to hermaphrodites using gametophytes with two different genetic backgrounds, wild type and *her1* mutants. Second, we will observe the release of sperm upon the addition of water and the chemotaxis of sperm toward the archegonia. Finally, you will break into groups to develop hypotheses and an experimental plan to test the molecular mechanism responsible for the altered sexual development observed in the *her1* mutants. You will set up these experiments in class today.

Procedure

I. Effect of density on sexual development. There are plates of wild type and *her1* plated at five densities each. The plates labeled 1:2 should have approximately 150 gametophytes total, while plates labeled 1:4, 1:8, 1:16, and 1:32 should have approximately 75, 38, 19, and 9 gametophytes, respectively.

Procedure

Remove the lid from your plate and place it under the dissecting microscope at the highest magnification. Notice the difference between **hermaphroditic** and **male gametophytes** by comparing against the figures provided. Note that the antheridia are very obvious in males.

Each person should select one wild type and one *her1* plate and determine the percentage of male gametophytes. If you choose one of the lower density plates (1:8, 1:16, or 1:32), you should count all of the gametophytes on your plate and determine the percent males. If you choose one of the higher density plates (1:2 or 1:4), you should count the number of gametophytes in 5 separate squares of the grids that are provided and determine the percent males. Pick the five squares at random before looking at the distribution of gametophytes on your plate.

Record your data in the following table **and on the class data sheet**. **For your lab report, determine the average percent male for both wild type and *her1* plates using the data from all 11 lab sections** (data available on Blackboard).

Density	**WT or *her1* (specify)**	**No. of Hermaphrodites**	**No. of Males**	**% Males**

II. Fertilization in C-Fern. Once the table has been completed, *each pair* should find one plate of wild type at the 1:2 or 1:4 density. Find a hermaphrodite with one or more males close by. Add a drop of water over the gametophytes and observe the release and chemotaxis of sperm under the 4x or 10x objective of the compound microscope. Very soon you will see sperm swimming and hopefully you will see that the sperm congregate at the archegonia.

III. Set up experiment to test mechanism of *her1* sexual development.

Scientific process

Involves four steps.

1. A scientist makes an observation.
2. A clearly defined question arises from that observation.
3. A testable hypothesis is developed to attempt to answer the question.
4. The scientist runs a carefully designed test, an experiment, to test the hypothesis. A well-designed experiment has several elements:

Elements of a well-designed experiment

The experiment will test the effect of **independent variable(s)**, which can be controlled, on a **dependent variable(s)**, which can be measured. In order to make sure that we are really measuring the effect of the independent variable, we must keep all other variables constant for each treatment; these are **control variables**. We must also make sure that the intensity of the independent variable is likely to cause a measurable effect. This is often not known precisely, so a range of intensities is used; these are the **treatment groups**. Additionally, we must use **replicates** of each treatment to assure the reproducibility of our results. Finally, you must include appropriate controls in which the independent variable is not varied; these are the **control groups**.

Example experiment

Observation: Dwarf pea plants contain a lower concentration of the hormone gibberellic acid (GA) than wild type pea plants of normal height.
Question: Does GA regulate plant height?
Hypothesis: Addition of GA to dwarf plants will allow them to grow to the height of normal plants.
Experimental Design:
-*Independent variable*: concentration of GA treatment added to dwarf plants.
-*Dependent variable*: height of plants.
-*Control variables*:

- duration of experiment.
- growth conditions for plants (temp., light, soil, water, fertilizer)
- quality of seed (i.e. all seed is of same age and is genetically similar)

-*Control groups***:**

1. Negative control: dwarf plants in the absence of GA – are expected to be short and will serve as a reference point for GA treatment groups.
2. Positive control: normal plants in the absence of GA – are expected to be tall and will serve as a reference point for GA treatment groups.

Procedure

A. Develop hypotheses regarding the possible molecular mechanism by which *her1* mutants are altered in their sexual development. Break into groups of 3 or 4 students. Take 5 minutes to develop a hypothesis with your group and then share these hypotheses with the rest of the class.

B. Develop an experimental design to test your hypothesis. In your group, take another 5 minutes to design an experimental plan. Your experimental design must describe the independent variables, dependent variables, at least three treatment groups and the appropriate control groups; most experimental designs should include both a positive control and a negative control. At the end of the 5 minutes, share your experimental plan with the rest of the class. Ultimately, several groups will carry out identical experiments in order to provide replicates of each treatment.

Materials:
It is relevant to your experimental design to know that the following materials are available. Note that the C-Fern cultures were plated 3-4 days ago and the gametophytes are at the perfect stage to respond to antheridiogen.

- Wild type cultures with population density 1:8.
- *her1* cultures with population density 1:8.
- Filtrate dilutions from wild type cultures 1:1, 1:3, 1:9, 1:27, 1:81.
- Filtrate from *her1* cultures 1:1, 1:3, 1:9, 1:27, 1:81

C. Carry out your experiment. You will measure your results one week from today and tabulate your results on a class data sheet. **Your data set will comprise all of the replicates for all 11 lab sections** for the exercise carried out today on the effect of density on sexual development as well as for the experimental data that you will collect next week.

References

1. Hickok LG, Warne TR, Duncan SB. 2004 C-Fern® Manual: Part B. Culture Instructions for C-Fern® Investigations. Carolina Biological Supply Company, Burlington, NC. Available from http://c-fern.org/index.php?option=com_virtuemart&Itemid=43 (accessed 2008 Feb)

Lab Report

Your lab report will consist of a **Title**, **Names of Authors**, **Abstract**, **Introduction**, **Materials and Methods**, **Results, Discussion**, and **References** sections. Consult Chapters 4 and 5 in Knisely's *A Student Handbook for Writing in Biology*, 2nd edition for instructions on writing these sections. All sections must be **typed double-space** (or use the template available at <www.sinauer.com/knisely>).

Descriptive Title and Authors

Abstract

Purpose of experiment
Brief description of methods
Results
Conclusions

Introduction

Background information on the mechanism of sexual development in wild type C-Fern (cite literature sources!)
Description of *her1* mutation
Objectives of your experiment

Describe the population density experiment (Week 1) as a preliminary experiment that you used to come up with your objectives for the experiment you designed (Week 2). Be sure to incorporate your hypotheses in this part of the Introduction.

Materials and Methods

All relevant information to enable reader to repeat experiment

Density of spores, strain of gametophyte, concentration of filtrate, temperature inside humidity dome, light source for culture, etc.

Results

- Text description of the population density graph (see next item).
- Plot the averaged class data from all 11 lab sections for both wild type and *her1* gametophytes for the population density experiment. See *A Student Handbook*, pp. 198-200 for instructions on plotting two lines on one set of axes. Plot the dependent variable [Males (%)] on the y-axis and the independent variable [Population density (# gametophytes/plate)] on the x-axis. Each strain (wild type, *her1*) forms a separate data set and should be identified using a legend. Make sure the figure is correctly captioned (figure number and title).
- Text description of the sex determination mechanism graph (see next item). Specifically, compare the % males in the test plates with those in the positive and negative controls set up for each hypothesis.
- Plot the averaged class data from all 11 lab sections on the experiment designed to examine the mechanism of *her1* sex determination. Plot Males (%) on the y-axis and Filtrate (%) on the x-axis.

Discussion

- Recap the relationship between population density and % males for each strain of C-Fern (Week 1 results).
- Explain *why* the percentage of males increased as population density increased for the wild type strain.

- Recap the results for Week 2. Specifically, what was the effect of wild type filtrate concentration and *her1* filtrate concentration on the % males in the test plates versus the % males in the positive and negative controls set up for each hypothesis?
- Explain the significance of the % males on each test plate compared to % males on the positive and negative controls for each setup.
- Using the class data, argue persuasively why the one hypothesis is negated while the other is supported.
- Cite at least one relevant, primary journal article that supports your conclusions. Write at least one paragraph on how other authors set up their experiment, the results they obtained, and the evidence that led them to come to their conclusions. Draw analogies between their findings and conclusions and yours.

References

Use the databases PubMed or Web of Science to locate appropriate references (*A Student Handbook*, pp. 12-15)

Use correct format for both the citation in the text and the complete reference (*A Student Handbook*, pp. 57-65)