Reproductive Biology of *Hackelia venusta* (Piper) St. John (Boraginaceae)

Norma Jean Taylor

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Abstract

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Norma Jean Taylor

Chair of the Supervisory Committee:
Associate Professor Sarah E. Reichard
College of Forest Resources

Hackelia venusta (Showy Stickseed) is an endangered plant consisting of a single population in Washington State, USA. This study is an examination of breeding system, pollination biology, seed germination, and container culture of plants to aid in recovery of the species. *H. venusta* is primarily outcrossing, but geitonogamous selfing may occur in open pollinated plants. Autogamous selfing is less likely, but may also occur. Three species of pollinators were confirmed during one field season: two Hymenoptera and one Dipteran. Seed counts were taken from a sample of the population for one season. A seed germination protocol was developed using seeds of a surrogate species, *Hackelia diffusa* var. *arida*, and *Hackelia venusta*. Plants were grown in containers, and suggestions for conservation actions, *ex situ* propagation, and culture are included.

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DEDICATION

To my parents Robert and Ruth Taylor,
my husband Thomas Lenon, and sons Alexander and Patrick Lenon

INTRODUCTION

The Washington State endemic plant, *Hackelia venusta* (Piper) St. John (Boraginaceae) (Showy Stickseed) is known from a single population of less than 800 plants in Washington State. It has been on the Endangered Species List since 2002 (US Fish and Wildlife Service 2002). On October 10, 2007 Federal and State agencies responsible for managing listed plants (U.S. Dept of Fish and Wildlife, Washington State Dept of Natural Resources, U.S. Forest Service) published the final recovery plan for restoring and extending self-sustaining populations in suitable habitat (US Fish and Wildlife Service 2007).

The recovery team previously considered risk factors to the population such as over-collection, effects of fire suppression, and damage from Department of Transportation activities (US Fish and Wildlife Service 2002). Interest is now directed toward questions of reproduction, and whether pollination and seed set might be limited, thus contributing to the apparent decline in number of individuals. The literature concerning the reproductive biology of *H. venusta* is not extensive. Details reported for reproduction are incidental to investigations of taxonomy and classification (Gentry and Carr 1976, Carr 1974), assessment of population numbers and habitat to determine official status (Gamon et al. 1997), or experiments that did not yield any data (Harrod 1999). There are no published experimental studies that address breeding system and pollination ecology, including observation of pollinator behavior, collection of voucher specimens of pollinators, experimental manipulation to assess the degree of selfing and outcrossing, counting seed set over several seasons, mean seed set in a season for baseline data, or a seed germination protocol. Carr's (1974) crossing experiments were aimed at interspecific crosses, and he grew seedlings by excising embryos and growing them under laboratory conditions. No germination methods have been documented using simulated natural conditions to investigate germination and dormancy.

My objectives were to establish some preliminary information about the breeding system, collect, identify, and observe as many pollinators as possible, develop a seed germination protocol, and to learn something about container culture to guide future propagation and reintroduction projects. I was also interested in measuring seed set, because it was believed to be low (Gamon et al. 1997)

CHAPTER I. BREEDING SYSTEM SYSTEM OF *Hackelia venusta*

Pollination Biology - Background

Pollen contains the gametes of the male parent that unite with those of the female parent. The study of gene flow in flowering plant populations is the study of pollen movement. Shivanna (2003) describes what occurs when pollen is transferred to a receptive stigma as "…a series of dialogues between the male gametophyte and the sporophytic tissues of the stigma and style." Stigmatic surfaces and pollen contain substances that enable them to interact, and enable pollen to survive desiccation. To successfully produce seed, pollen must adhere to the stigma, hydrate, and germinate; the pollen tube must enter and grow down the style, and reach the ovary to deliver the male gametes that unite with eggs in the ovary to achieve fertilization (Shivanna 2003). Pollen tube growth is affected by the phenotypic expression of the pollen genotype, and (to a greater or lesser degree, depending on species) by female sporophytic tissues that allow germination, growth, and fertilization of certain pollen preferentially; this effect not only differs from species to species but sometimes from plant to plant within a population (Schlichting et al. 1987, Lyons and Antonovics 1991, see also Fenster 1991a for a review). The progress of pollen tubes can also be affected by the *amount* of pollen deposited on the stigma, because pollen tube competition can stimulate growth, with the faster growing tubes reaching and fertilizing the ovary first. This in turn affects the genetic structure of the population (Schlichting et al. 1987, Kearns and Inouye 1993).

The term *pollen quality* may refer to the viability and vigor of pollen (Towill 2004) or, in studies of population structure, to the source or genetic relatedness of pollen that arrives at the stigmatic surface. In hand pollination experiments, the viability of pollen used to manually pollinate flowers is important, and the quality of pollen in this sense is affected by maturity, freshness, the timing of collection, and quality control in storage conditions (Stone et al. 1995). There are several ways to

test for pollen viability (Shivanna and Rangaswamy 1992, Stone et al. 1995, Towill 2004).

In the context of pollen quality by source, plants exhibit all levels of compatibility from complete selfing, through facultative selfing, facultative outcrossing, and complete outcrossing, so pollen accepted (and therefore pollen quality in a genetic sense) differs with breeding system. To use an outcrossing species as an example, higher quality pollen would be pollen from another plant. Outcrossed pollen tubes might grow faster and out-compete self-pollen in the style. If male gametes from closely related individuals reach and fertilize ovules, selective seed abortion by the female parent may later decrease seeds that are inbred and less fit (thus avoiding inbreeding depression).

Experiments using hand pollination must employ methods to assess the timing of stigma receptivity to ensure that pollination and fertilization can occur. As with pollen viability tests, there is more than one method to test for stigma receptivity (Galen and Plowright 1987, Dafni and Maues 1998). Choice of test may depend on the number of plants, time available, and plant species. The flowers in question may have to be tested to calibrate the particular species-specific response (Galen and Plowright 1987).

Often, outcrossing species will have mechanisms for avoiding the deposition of self-pollen on stigmas in the first place, because it may cause pollen clogging or pollen discounting, i.e. wasted pollen that does not fertilize ovules thus interfering with higher quality pollen reaching the stigma. Mechanisms that separate anthers and stigmas on plants in space (herkogamy), or in time (dichogamy) are ways of avoiding self-pollination because pollen contact is less likely (Barrett 2002, Shivanna 2003). Protandry (anthers mature before stigmas are receptive) and protogyny (stigmas receptive before pollen sacs dehisce) are common ways of keeping self pollen out of the pollination process for hermaphroditic flowers that have essentially identical morphology on all plants.

The degree to which self-pollen is able to fertilize facultatively outcrossing plants may change during the season, and varies from species to species. In species that are protandrous, for example, there is more pollen available early in the blooming season. As pollen is depleted and less is available, female choice may then allow more self-pollen access to ovules, resulting in an increase in selfed seeds later in the season (Galen et al. 1985, Jennersten et al. 1988, Fenster 1991a, Melser et al. 1999, Melser and Klinkhamer 2001). Later pollinations may result in lower seed set because these later seeds are more often inbred and therefore are aborted more often (late-acting inbreeding depression), or finite maternal resources may be preferentially allocated to seed formation from prior pollination events regardless of pollen source. Bertin (1985) found that even when pollen was added by hand, fruit formation in *Campsis radicans* was limited if plants were already ripening fruit. He concluded that fruit formation was limited by the interaction of both pollination timing and resource allocation. Jennersten et al. (1988) found that these factors together with fewer ovules available during the latter part of the blooming period caused seed production to vary over time. Interaction between male gametes and the female sporophyte reflect the evolutionary development of a particular species (see Barrett 2002 for a review). This is why pollination studies remain a valuable tool to study plant population ecology.

To study pollinators' role in plant reproduction, flowers are covered with bags to exclude insects and gauge the effect on seed set compared to open pollinated, unbagged flowers. Anthers or styles can be removed on plants with large, accessible flower parts to prevent self-pollination and isolate those plants that are only pollinated by insect visitors or by wind or water (Kearns and Inouye 1993). Field observations are confirmed by hand pollination, either in the field or in containers in a nursery or greenhouse where plants can be manipulated more easily with known quantities and sources of pollen.

In-depth pollination studies observe the growth of pollen tubes in styles to confirm pollination, and record competition between self and cross pollen, count

pollen grains on insects and pollen grains per stigma, and use powdered dyes to track pollinator movements to determine how effective a particular pollinator may be and how far the pollen is carried (Kearns and Inouye 1993). Seed obtained from pollination treatments is germinated for an assessment of the success of a particular breeding system. Viable seed should germinate, but it is important to also assess seedling survivorship and reproduction to measure the health of plants produced. The genetic lines and number of individual plants that survive in a horticultural setting will be different than those growing in the field (S. Reichard pers. comm. 2004). Mortality may be higher in the field, or higher in containers. For the purpose of plant conservation, seedlings of known parentage should be tracked in the field to measure the effects of selfing and outcrossing. The aim of my pollination experiments was to answer the basic question of whether *H. venusta* is selfing or outcrossing. I used potted plants for hand self and outcross pollination and bagged plants in the field to measure the effect of open pollination and pollinator exclusion on seed set.

Breeding System Study

Site description

Hackelia venusta occurs at an altitude of 1550 to 2700 feet, just east of the crest of the Cascade Mountains in Chelan County, Washington (Washington Natural Heritage Program 2004). Because of the proximity of the site to the Cascade crest, the climate is influenced by westside maritime air, as well as elevation, and is moister than the shrub-steppe vegetation zone to the east. Monthly average low temperatures for the Leavenworth area are below those of Wenatchee, and precipitation is higher (Western Regional Climate Center 2008). Soils at the Tumwater Canyon population are described by Gamon et al. (1997) as "loamy sand or sandy loam with 0 to 40 percent gravel…derived from granitic and gneissic rocks" on a slope of 25 to 70 degrees. Plants I studied also grew on steeper slopes, and those in rock crevices up to 90 degrees were among the largest I worked with. Plants at higher elevations in the population can be seen growing in inaccessible cliff tops or cliff faces; large plants also grow among grass, and under the shade of large conifers. The texture of the surface soil ranges from sand to coarse gravelly sand that is easily displaced, especially when it dries out during the summer. The site as a whole is so steep that loose material or objects on almost any part of the site readily roll downhill. Cliffs provide protected sites for plants, but are also the source for loose slabs of rock and boulders that are constantly sliding downhill and threatening established plants. In 2004 and 2005 snags left over from a previous wildfire were beginning to fall and shed branches, and one live Douglas fir dropped branches on and around a groups of *H. venusta* plants in the study.

Slopes face west and south. In spring and summer the slopes are fully exposed to the sun around mid-morning (10:30 am approximately); south facing locations are often in shadow by 4:00 pm; west facing slopes are in the shadow of cliffs and more distant mountain ridges about an hour or two later, depending on local topography.

The average annual low temperature (17.8 C) occurs in January, and the average high (30.9 C) in July. Greatest average snow depth is 51 centimeters in January, mostly melting by April (Western Regional Climate Center 2008, pers. obs.).

Flower Morphology

Gentry and Carr (1976) describe the inflorescence in the genus *Hackelia* as "a usually paired, helicoid cyme…sometimes referred to as a scorpioid cyme, sympodial false raceme, or a panicle." Following Zomlefer (1994) the tightly coiled, circinate inflorescence is a scorpioid cyme, with flowers opening alternately along the axis of the inflorescence, beginning at the bottom of the peduncle. As flowers mature, the peduncle straightens and elongates. Flowers open one by one, with two to four fully open at one time (Figure 1.1). When most of the flowers have opened, the older, lowermost have begun to form green fruits. Often the last one or two buds do not open or if they do, do not form seeds.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Figure 1.1 *Hackelia venusta* in bloom, field site *J. Taylor*

Figure 1.2. Section showing mature stigma and anthers *J. Taylor*

Although upper corolla lobes are showy, the lower petals are fused into a tube, the opening almost completely obstructed by epipetalous fornices (appendages), and the reproductive parts are entirely enclosed and concealed. A bright yellow, central spot of color marks the entrance to the tube. The five epipetalous anthers, one per petal and attached below the fornices, extend in toward the center of the tube. The pistil is below the anthers, and elongates as it matures until it almost contacts the anthers (Figure 1.2). There are four, or occasionally six, ovules around the base of the style. Two carpels normally contain four ovules, but I observed extra ovules more than once in flowers in the field and on potted clones. I never observed six nutlets from one flower, however. The fruit is a schizocarp that splits into four nutlets, although these are easily identified as four separately forming nutlets during all stages of bloom.

Phenology

Hackelia venusta begins to bloom in mid to late April and bloom ends in June approximately seven to eight weeks later. A few scattered late blooming plants can be in full bloom as late as the end of June and some plants will re-bloom in October, depending perhaps on temperature and moisture (pers. obs.). While one flower is opening, one or two others above it on the same cyme will begin to open consecutively. At full bloom a large branch can have several flowering cymes, with seeds forming on the lowest flowers.

Flowers are protandrous (anthers dehisce along a seam-like suture and pollen is available before stigmas appear receptive). Anthers are smooth and light- colored when flowers first open, and within the first day or two, split open exposing the pollen. They occasionally may dehisce as the flower opens, but always very early in the flowering cycle. I have observed that the pollen sticks together, perhaps because of specialized threads [see Hesse et al. (2000) for a discussion] or pollenkitt, a substance produced with pollen. This may make it less likely for the pollen to rain

down on the stigma. As the anthers age, they dry and brown around the open edges, opening inside-out to present pollen that appears rough and yellow on the surface.

The pistil does not necessarily ever contact the anthers. When the flower opens, the pistil is light green, very smooth, and about 0.5 mm high. As the flower ages, the stigma darkens to a yellowish color, the surface lobes become more pronounced, and stickier. The pistil elongates to a maximum of about 1.5 to 2 mm - about 3 times its original length (Figure 1.2). As the flower ages, the corolla wilts and collapses around the stigma briefly, then drops off in one piece. The naked stigma is exposed, surrounded by the sepals, which stay stiffly upright, persist, and then reflex as the stem ages and seeds form (arrow Figure 1.1).

Flowering proceeds as follows: 1) tight green bud on tightly curled cyme; 2) tight white bud; 3) loose white bud (petals slightly separated, but still curled); 4) flower open, with petal lobes flat, anthers smooth; 5) anthers dehisce and style begins to elongate; 6) anthers brown, pollen fully exposed or removed, style reaches maximum length, stigma lobes pronounced, sticky, and darker in color (may be yellow or brown if damaged). Nectar flow occurs in the disc at the base of the style while the flower is open (I did not record timing or quantity of nectar); 7) Corolla wilts, petals drop approximately three days after stage 4.

Carpels at the base of the style appear green and plump after petal fall, and begin to develop characteristic prickles before seeds are filled. A week or two before nutlets mature, unfilled seeds shrivel up, sometimes after beginning to look full. It is not possible to assess the actual number of seeds that will finally form until they are almost ripe, just prior to dispersal, because seeds are aborted late in the process. It is common to find flowers with 0, 1, 2, or 3 seeds. This will be discussed further in the section on seed crop. When ready to disperse, seeds are dry and easily dislodged. They may disperse individually, or as a unit of 2, 3 or 4 attached to the style, more commonly individually. Seed size is not an indicator of germinability (see Seed Germination Protocols Chapter III). At his point the stems are brittle and may break off while still holding some seeds. I observed that once the seeds dispersed, it was

often possible to tell if an individual flower produced any seed by checking to see whether the dried sepals were reflexed. There may be a smooth area where the seed or seeds fell off as well. If the dried sepals remain upright around the empty center it is likely that no seeds formed on that flower.

In the fall of 2004, some plants in the field bloomed a second time after rain in October. Potted plants responded to increased moisture as well, but only the largest formed buds in the fall. With cold weather approaching, it is unlikely the late blooms would have time to mature seeds even if pollinators were active.

Materials and Methods

Hand Pollination

Potted plants representing eight genetic lines raised by tissue culture (multiple clones per genetic line) were available for hand pollination experiments in 2004. Because some individuals are easier to clone and to grow in containers than others, there were many more plants of one clone (#20) than others. Eighteen of the thirty-five plants were from this one genetic line; other lines were represented by one to four plants each. Some of the plants did not live to the end of the summer. Pots had been overwintered near Leavenworth, Washington to expose them to natural winter weather conditions, and were transferred to Seattle in April. The plants were kept outdoors under a shade cloth during the growing season.

The blooms on potted plants were smaller and easier to damage than those in the field. Since individual stems and branches were too fragile to attach bags directly to the plants, I wrapped bridal veil mesh around each pot to exclude pollinators and control pollen movement among flowers. The following year (2005), very few plants survived the winter, and there were not enough flowers for hand pollination.

As flowers opened, I assessed pollen maturity by looking inside a flower or removing petals to look for anther dehiscence. I used pollen from fully open flowers and fully dehisced anthers. I judged pollen to be mature when anthers were open and

pollen grains could be easily seen (anthers developed a rough appearance). I used a small dropper to place 3% hydrogen peroxide on stigmas of open flowers to test for receptivity (Kearns and Inouye 1993). The results of the test were not conclusive. I observed a bubbling reaction on stigmas at all stages of development. On older stigmas, i.e. those at full length, with surface lobes and stickiness most apparent, hydrogen peroxide produced a substantial reaction on the tip after one minute. I eventually used visual inspection to judge receptivity, as the stigma lobes became more pronounced and appeared stickier later in development, with possibly increased nectar at the base of the style. Occasionally I applied pollen to stigmas based on the availability of the pollen and a stigma on a suitable clone, rather than on receptivity. This was because pollen was in short supply, it was unclear how long stigmas would survive after removal of the petals for pollen transfer, and flowers were open for such a short time (average about 3 days).

I experimented with pollen collection using small toothpicks and cotton, but these methods damaged the flower parts, and wasted pollen because it was hard to remove from the utensil. The best way to see and manipulate the flower parts was to place the pot under a dissecting microscope and use a pair of tweezers to remove the entire corolla (the corolla is easier to remove after the flower has been open for one day). I then grasped an individual petal with anther attached, detached it as a unit using the petal as a handle and rubbed the anther with exposed pollen gently on a stigma. This technique prevents damage to the stigmas while applying adequate amounts of pollen (Figure 1.3).

I applied pollen from a flower to either the same flower (autogamous selfing), another flower on the same plant (geitonogamous selfing), or another plant of the same clone (geitonogamous selfing) or a different clone (outcrossing). Depending on how many stems flowered and which flowers were open, I was able to apply all three treatments to some plants, but others received only one or two treatments. For autogamous selfing, I plugged the corolla with a piece of cotton ball or some bathtub caulk to exclude any outside pollen and left the flower to self naturally.

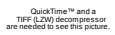

Figure 1.3 Hand pollination *Peter Haley/The News Tribune*

I observed aphids feeding inside flowers, even in pots covered with a double layer of netting. Aphid damage on potted plants in Seattle was extensive on the leaves and readily apparent on flowers, where they were inside the corolla tube feeding on styles or nectaries. They were only a problem on plants in Seattle - I have not seen any in the field population. Thrips (Thysanoptera) were abundant and active in 2004. I captured adult thrips and larvae on potted plants and plants at the field site. They appeared to be using the flowers as a brood site for larvae; adults probably feed on pollen without damaging the flowers (Kirk 1984a, 1985, Sakai 2002). No thrips damage was observed, and I saw very little insect damage of any kind in the field.

In 2004, I hand pollinated the first flowers on April 17 and the last on May 12. In June I collected and stored ripe seeds in the Miller Seed Vault, at the Center for Urban Horticulture, University of Washington.

Field Bagging

The Washington Natural Heritage Program (2004) designated the most well-known population the "main" population. This population of approximately 300 is in a fragile, unstable habitat. A survey conducted in 2004 found "subpopulations" A, B and C (approximately 450 plants altogether) to the south and east of the main population, that were on firmer ground and could be approached more easily. (The main and subpopulation designations are somewhat arbitrary, reflecting the order in which they were found, and I use the names as a convenience rather than to denote any genetic relationships. Each subpopulation is spread over an east-west altitudinal gradient that might better be used to note the trajectory of gene movement or seedling establishment from higher to lower elevation.) The fragility of the landscape and rarity of the plants precluded random selection of treatments and controls in the field. In 2005 I searched for the maximum number of plants over as wide an area as was practical. I chose plants that could be repeatedly visited without causing too much land slippage or erosion. Several plants in cliff faces would have presented good subjects but were impossible to reach. I chose fourteen plants of various sizes throughout the subpopulations.

I observed plants and collected some insects in the field in 2004. Fieldwork on pollination and breeding system took place mainly in 2005. It became apparent from my potted plant experiment that it is important to be aware of mesh size and the integrity of the bag to exclude very small insects. When choosing material for field experiments, I took into account exposure to sun and the elements, and difficulties Harrod (1999) had in keeping bags on plants in the field. I constructed bags with seams that would have the smallest effect on the climate around the bagged stem (see discussion in Kearns and Inouye 1993) and still provide a strong barrier that would hold up to sun and weather.

To exclude larger insects such as bees and flies, I used very fine mosquito netting (UV treated No-See-Um Netting, white, part # IN-008 from Barre Army/Navy Store, 955 N. Main St. Barre, VT). I observed many thrips in 2004, and

had such a problem with aphids on potted plants that I thought it important to make a second set of bags from special netting used by greenhouse growers to exclude minute insects (NO-THRIPS™ manufactured by Green-Tek, supplied by international Greenhouse Company Item # IS-NT39). This product has a mesh size that is smaller than the narrowest part of the head of an adult thrips I collected (0.152 mm).

I constructed bags of different lengths to accommodate short, curled inflorescences early in the blooming season, and changed them as flowering stems elongated and formed seeds. I folded over and sewed a casing on the open end, inserting a length of doubled thread so the bag could be tightened easily around the stem. The other three sides were sewn shut. (Figure 1.4). I sometimes used bags with no casing, and tied them on the outside of the bag, because this reduced the amount of stiff material bunched-up around the relatively weak stem. A second set of bags for seed collection were open at both top and bottom so they could be slipped over the stem more easily when the first seeds began to ripen, left open to allow pollination of the remaining flowers, then unrolled up the stem and closed as the last flowers faded. I measured seed production on open pollinated stems of *H. venusta* and a small group of *H. diffusa* var. *arida* in the same habitat. I counted seeds on control (open pollinated) stems on the plants I had used for the *H. venusta* pollination study, and added seed collection bags to an additional eleven untreated plants in each subpopulation. I also put seed collection bags on eleven stems on a small patch of *H. diffusa* var. *arida* in one of the subpopulations of *H. venusta*. The bags were positioned to allow pollinator access to all flowers; I gradually covered developing seeds on the lower part of the stems, and closed bags completely after the last flowers wilted. I left seeds from untreated *H. venusta* at the site near the parent plants.

Plants had from 4 to 81 individual stems and from 8 to 96 flowers per stem. I located plants at the beginning of bloom and bagged two stems as treatments, one with a mosquito-net bag and one with a thrips-excluding bag. I chose a control stem

for each treatment plant later as seeds began to form, and put a loose bag on that stem to catch seeds before they fell to the ground, but did not cover flowers still in bloom. Stems had to be chosen for all treatments and seed collection based on their ability to hold a bag, and some plants were quite small, so the choice of stem was not random. I returned approximately once a week to check on the bags, adjust them, and change to larger bags when necessary. As seeds formed, I closed up each seed-collection bag individually after all flowers were wilted and allowed seeds to ripen before collecting them. Seeds were kept separate by plant of origin. I made the last seed collection on July 24, 2005.

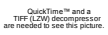

Figure 1.4 Thrips and mosquito net pollination bags *J. Taylor*

Results

Hand Pollination of Potted Plants

Both aphids and thrips had access to flowers on potted plants in Seattle. I applied the insecticide Talstar ® to the plants to kill aphids and thrips, and I was able to control the insects that were contacted with the spray, but did not continue to spray because I did not wish to damage the remaining flowers. Aphids feeding inside flowers could easily have spread pollen between or within flowers. A few flowers had self pollen on the stigma when I first opened them for hand pollination, although no insects were present, so these could be presumed to be instances of unfacilitated autogamous pollinations (Lloyd and Shoen 1992). I was able to exclude bees and larger flies.

Table 1.1 summarizes seed set and germination for hand pollinations. Only four groups of clones (numbers 20, 21, 29 and an unlabelled unknown) were responsible for all seed. I did not track flowers on individuals, or on untreated bagged and unbagged plants, for per-plant seed set percentages, but only pollen transfers by clone number. Geitonogamous selfing and outcrossing produced the highest seed set (4.5 and 3.3 percent, respectively). Single-factor Anovas performed for both seed set and seed germination did not show significant differences (among crossing and selfing treatments $F= 0.362$, $P=0.701$; germination across treatments $F=0.555$, $P=0.672$). Seed set percentages are based on potential seed set of 4 per flower. Some plants in the natural population and in pots had six ovules. However, the assumption of four ovules was used because it was not possible to count them on every flower, and six ovules is uncommon. Two of five untreated bagged plants produced seed; two unbagged open pollinated plant did not produce seed. Germination was highest for the autogamous selfed seed, but the sample size was very small (2 seeds). Over all treatments seed germination was 42.2%.

Table 1.1 Seed set and germination percentages for hand pollinated clones

Clone# 21	**X**	**S**	**SS**	**Bag/No Trt**	**Open Poll**	**Total**
Flrs treated	28	3	2	0	0	**33**
Seed Set	6 (5.36%)	2 (16.7%)	0			**8 (6.1%)**
Seed Germ	6 (100%)	0				**6 (75%)**
Clone# 20						
Flrs treated	14	28	14	4 plants	2 plants	
Seed Set	1 (1.8%)	5 (4.5%)	2 (3.6%)	24 No flr count	0	**32**
Seed Germ	0	3 (60%)	2 (100%)	5 (20.8%)		**10 (28.1%)**
Clone# Unkn						
Flrs treated	3	0	0	0	0	**3**
Seed Set	4 (30%)					**4 (30%)**
Seed Germ	3 (75%)					**3 (75%)**
Clone# 38						
Flrs treated	7	3	3			**13**
Seed Set	0	0	0			**0**
Clone#35						
Flrs treated	6	1	1	1 plant	1 plant	**No count**
Seed Set	0	0	0	0	0	**0**
Clone 33						
Flrs treated	0	1	2			**3**
Seed Set	0	0	0			
Clone# 29/38(no label)						
Flrs treated	2	2	0			**4**
Seed Set	0	0	0	0	0	**0**

Table 1.1 Seed set and germination percentages for hand pollinated clones, *continued*

Clone# 29	X	S	SS	Bag/No Trt	Open Poll	Total
Flrs treated	18	0	0	0	0	**18**
Seed Set	1 (1.4%)					**1 (1.4%)**
Seed Germ	0					**0**
Clone# 28						
Flrs treated	11	1	0			**12**
Seed Set	0	0				**0**
Clone# 17						
Flrs treated	1	0	0			**1**
Seed Set	0					**0**
All plants						
Flrs treated	**90**	**39**	22	**5 plants**	**3 plants**	**83 + 8 plants**
Seed Set	**12 (3.3%)**	**7 (4.5%)**	**2 (2.3%)**	**24**	**0**	**45**
Seed Germ	**9 (75%)**	**3 (42.9%)**	**2 (100%)**	**5 (20.8%)**	**0**	**19 (42.2%)**

Key: S = geitonogamous SS = autogamous X = cross Bag/no trt received no hand poll. Open poll had no bag.

Field bagging

In the field population, fourteen plants were originally treated for this study. Some bags were lost, either because they fell off, or were removed by animals, or the stems were broken with the bags on and were then lost. Bags made from NO-THRIPS ™ damaged stems more often because they were stiffer and heavier. Getting these bags off for flower counts risked damage to the stems, so I did not count flowers on all stems for this treatment and consequently do not have seed set percentage data for these stems. A large tree branch fell on one plant and it was damaged as I attempted to extricate it. A total of three control stems died. One whole plant died suddenly with a full, partially ripe seed crop, affecting all treatments on the plant. It was not possible to tell how many seeds would have ripened, but four of

eighty-one seeds on the open pollinated control stem did germinate, so this control stem was included in the total seed count. Four mosquito net treatments and six thrips net treatments died during the study.

A rainy spell during peak bloom caused damage to some flowers because the wet flowers were crowded together inside the bags. The effect of approximately two weeks of cloudy, rainy weather was evident in a gap in seed set on almost all plants in the population for this period whether or not they had bags. Flowers inside bags bloomed slightly longer than unbagged stems, indicating that the climate inside the bags was probably somewhat cooler, owing perhaps to the white bags reflecting some heat and possibly increasing humidity. The difference was noticeable but not extreme.

I found one small fly (Diptera) in one mosquito net bag (one seed on the stem), and two parasitic wasps and an ant in three mosquito net bags (no seeds); one mosquito net bag end became loose and was open, but remained folded over (one seed in bag). Red mites (Acarina) were observed inside bags and on flowers in 2005. These bright red, fast moving mites were likely predatory. Small insects and mites may have been able to move a small amount of pollen inside the bags.

I collected bags when seeds were ripe in July. The seed set data in Table 1.2 show that the seed set on unbagged control stems was far above that of bagged treatments, and similar to plants in the nearby untreated population (26.5 percent for controls and 22.58 percent for plants chosen in the population for seed counts only). Seed set on bagged stems was less than two percent for larger mesh netting and also very low for thrips-excluding netting. Data analysis (Table 1.3) indicated that neither the means nor the variances were equal for seed set on bagged (mosquito netting) and control stems ($F=79.21$, $P=0.0001$ for variance; $t=6.126$, $P=0.0001$ for means). Percent seed set for thrips netting treatment could not be calculated because I could not remove the bags to count flowers and replace them without damaging the plants with these stiffer bags.

Table 1.2 Seed set on bagged and control *H. venusta* stems – field experiment

Treatment	n (# of Stems)	# of Flowers	# of Seeds	Mean Seed set per plant (potential seed = # of flowers X 4)	% Germination
Mosquito net	11	427	22	1.75%	0.09
Thrips net	8	na[1]	15	na	0.067
Control stem unbagged	12	505	494	26.5	14.57
Untreated plants	11[2]	336.5	304.5	22.58	n/a (sds left at site)

[1] Flowers not counted
[2] On one plant two stems were bagged together and the total divided by 2

Table 1.3 Treatment effect on seed set (field plants) – statistical summary

Treatment	n	Mean seed set	Variance	Test statistic	Crit value	P
Open pollinated	12	26.50	0.0193	F = 79.21	$F_{.05\ (2)\ 11,9}$ 3.66	<0.0001 (variance)
Mosquito net	11	0.018	0.0002	t = 6.126	$t_{.05\ (2)\ 11}$ 2.201	<0.0001 (means)

Table 1.4 illustrates seed set comparisons for *H. venusta* study plants and untreated plants nearby. The variances were equal. A two-tailed t-test with pooled variances showed no significant difference between the means. Mean seed set for the study population of *H. venusta* was 26.5%, whereas *H. diffusa* var. *arida* in the same habitat had a significantly lower mean seed set of 13.47% (Table 1.4, 1.5).

Table 1.4 *Hackelia venusta* and *Hackelia diffusa* var. *arida* seed set mean and variance.

	***H. venusta* study plants**	***H.venusta* untreated plants**	***H.diffusa* var. *arida***
n	12	11	11
sample mean seed set	26.50%	22.58%	13.47%
sample variance	0.0193	0.0155	0.0035

Table 1.5 Comparison of sample variances and means for seed set on *H. venusta* open pollinated stems and *H. venusta* compared to *H. diffusa* var. *arida*

	variance	mean
*H. ven*usta study plants X *H. venusta* untreated pl.	F obs= 0.804	T obs= -0.709 (pooled var)
	$F_{0.05\,(2)\,11,10}$ = 3.66 P = >0.50	$t_{.05(2)\,21}$ = 2.08 P = 0.486
H. venusta study plants X *H. diffusa* var. *arida*	F obs =5.528	t obs = 2.967
	$F_{0\cdot05\,(2)\,11,10}$ = 3.66 P = 0.012	$t_{0.05(2)21}$ = 2.131 P = 0.010

Seeds from study plants were tested for germination, which is one measure of viability. At the beginning of germination testing, after imbibing water and being chilled for two weeks, these seeds dried out because of an error in programming the incubator. Therefore the germination test for field plants was not ideal, and should be repeated if possible. Seeds germinated over an extended treatment period, but to a much lower rate than expected, based on previous germination trials with the surrogate species and hand-pollinated clones. Total germination was 75 seeds or 14.12% of the total collected from all stems; 14.57% of seeds from control (open pollinated) stems, 0.09% from stems with mosquito netting, and 0.067% from stems with thrips netting germinated during this time. Even though this germination test was not ideal, it indicates that open-pollination produced more seeds that germinated

at a higher percentage than plants treated to exclude insects. Figure 1.5 illustrates differences in per-plant germination for seeds from the field.

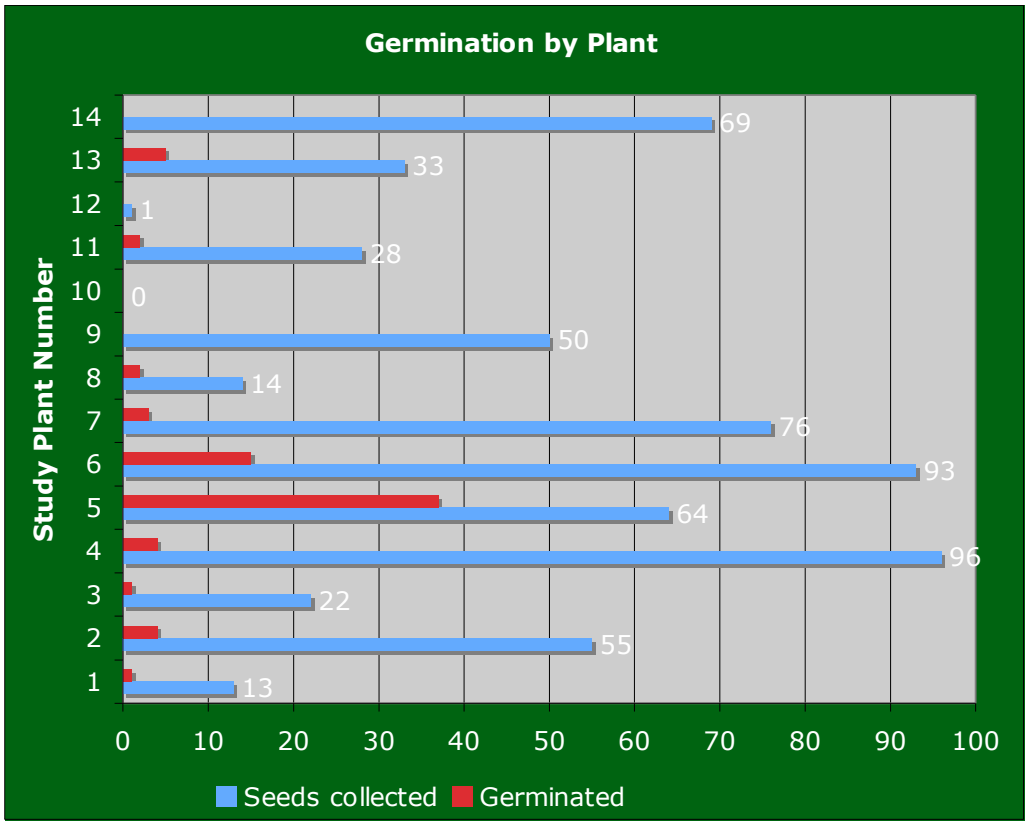

Figure 1.5 Germination rates and timing, *Hackelia venusta* field study

Discussion

Hand pollination yielded higher seed set on outcrossed flowers and those with pollen applied from the same plant but not the same flower (geitonogamy). Relatively high seed set on hand pollinated plants bagged to exclude insects, together with a 20.8% germination rate, do not necessarily indicate high autogamous selfing because the netting did not exclude thrips, aphids, and perhaps some very small flies that could have affected pollination rates. For the same reason, it is not possible to say for certain that seed set on hand pollinated, selfed plants was always the result

only of self-pollen. I was able to construct much better bags for the field experiment, and I am confident that those bags did exclude most small insects and virtually all larger ones, such as bees and flies. The results from field tests indicate that this species is predominantly outcrossing, relying on insects to deliver pollen most of the time. *The amount of insect-mediated selfing has yet to be determined, however.* The role of small and large insects in pollination is discussed further in the section on pollinators.

Adding pollen by hand, as opposed to insect mediated or spontaneous selfing, can show that hermaphroditic plants are capable of selfing, even though it may not readily occur naturally because pollen loads are too small, or because self-compatible, outcrossing species have evolved ways of avoiding it. Hand pollination is often used to test or confirm hypotheses about breeding system because the timing and amount of pollen is usually more controllable either in the field or in a facility where plants are contained. This is not the case with *H. venusta* – plants are difficult to raise in containers, they are smaller and weaker, and manipulation of flowers and pollen is difficult because of flower morphology and small, delicate, and concealed reproductive parts. It takes an appreciable amount of time to learn to manipulate plants for hand pollination. For these reasons natural seed set has been more successful.

Sample sizes for hand pollination were unequal because flowers had to be chosen as they opened, and it was unclear how many flowers from which clones would be available. Data from hand pollination experiments were affected by trying out different methods to get pollen onto stigmas without damaging them. Even when the method worked well, the condition of the stigmas suffered because they are normally entirely protected inside the corolla tube where humidity is higher, and are easily damaged when handled. The easiest way to hand pollinate individual flowers is to tear open the corolla, remove a petal with attached anther, and rub it across a stigma. Anthers are attached closely to petals, and might be clipped along with the

top part of the petal, to remove pollen from individual flowers but I did not judge this to be any better than taking the corolla off as a unit since it comes loose quite easily.

Altering the flower to get access to anthers and stigma changes the appearance of the flower to pollinators and the mechanics of aging. The petals will not age and wilt around the stigma naturally, and because *H. venusta* is protandrous the stigma would not necessarily be receptive when anthers are removed. Since a stigma is exposed when the corolla tube is taken apart, it seems this would prevent its normal development on flowers from which anthers had been removed.

The technique I used for autogamous pollination may have affected the result, since I rarely removed the petals and applied pollen to the stigma on the same flower, but rather closed up the corolla opening so that no pollen could enter from another flower. I judged that the proximity of the stigma to the anthers would be adequate for autogamous pollination, but it may also require an agent to actually move the self-pollen to the stigma.

I had intended to refine my techniques following the first year and to use a more accurate and simple measure of stigma receptivity (Dafni and Maues 1998). Few plants survived the winter in pots, and I did not have enough flowers for a second round of hand pollination. Hand pollination in the field was not attempted. It was difficult to tell when stigmas were receptive using the hydrogen peroxide test and, as Galen and Plowright (1987) reported, stigmas may test positive from the time they are first receptive, but older stigmas continue to test positive, so this is a qualitative measure that must be compared to actual fertilization events for accuracy. Damaged stigmas will also show a reaction to hydrogen peroxide. Therefore one must have enough stigmas available to destroy in the testing process and still have adequate numbers for pollination. Ideally, receptivity should be calibrated using several measures so that a mature stigma can be recognized by appearance.

The very low seed set from hand pollination, then, is the result of a combination of factors. Harrod (1999) reported no seed set from hand pollination, quite likely because of the difficulties discussed above. The primary benefit of this

hand pollination work was developing an understanding of the mechanics of hand pollination, flower phenology, stigma receptivity and flower morphology. If hand pollination is repeated, particular attention should be paid to pollen quality and viability, and if fresh pollen is not used, it should be tested to control for quality. Since the pollen is sticky, there may be challenges to collecting and handling it for storage and re-use. Consideration should be given to possible loss of viability if pollen is dessicated during storage and re-hydrated (Shivanna and Rangaswamy 1992, Stone et al. 1995, Towill 2004). The importance of accurate determination of both pollen viability and stigma receptivity cannot be overemphasized. Pollen tube germination may or may not correspond closely to stigma receptivity (Galen and Plowright 1987), but it is important to know when pollen may adhere better, and when stigmas are most likely to allow pollen tubes to grow. It is equally important to know how long pollen is viable at varying temperatures and humidity, because there may be a window of only a few hours during periods of high heat and humidity when pollen remains viable (Shivanna and Rangaswamy 1992, Stone et al. 1995, and references therein; Shivanna 2003). As previously noted, tests must be devised to map the progression of both stigma receptivity and pollen viability so that each can be handled appropriately to give accurate results.

The much higher seed set in unbagged plants from field experiments indicates that pollen movement by insects is necessary for most fertilization and therefore outcrossing is the primary breeding system, with perhaps some geitonogamy or autogamy facilitated by insects. The relative amount of seed set from geitonogamous selfing and outcrossing in the field remains to be measured.

Members of the Boraginaceae are known to ripen fewer than 100% of their seeds, and some are routinely as low as 25%. Melser and Klinkhamer (2001) report 25% seed set (not pollen-limited) in *Cynoglossum officinale*, a species in the Boraginaceae with very similar phenology to *H. venusta.* Goulson et al. (1998) measured seed set in *Symphytum officinale* (comfrey) in its native range at 1.18 per flower out of a possible four nutlets, also not pollen-limited. This is a robust,

common plant native to Europe and Asia, used in herbal medicine and widely introduced in other parts of the world. In a study on nectar robbing, Morris (1996) reported average seed set in *Mertensiana paniculata* (located in the Wrangell Mountains in southcentral Alaska) at 1.56 to 1.28 nutlets, and at most 2.31 when outcross pollen was added by hand. In a study of pollination and breeding system for the high altitude alpine plant *Eritrichium nanum,* Zoller et al. (2002) report that one to two nutlets per flower develop, usually only one. *E. nanum* also has very similar phenology, nectar production and flower morphology (including epipetalous fornices, protandry, and size and timing of style elongation) to *H. venusta,* although the plant itself is a smaller cushion plant and has blue flowers. Studies of *Echium vulgare* by Melser et al. (1999) and Klinkhamer et al. (1994) suggest that production of only one to two seeds per flower for this species may be the result of early and late-acting inbreeding depression. Seed set appeared to be genetically controlled in progeny, and fitness experiments with controlled crosses showed the importance of studying both male and female contributions.

Early in seed development on both *Hackelia* species, most seeds look normal until they are almost fully ripe and turning brown. Aborted seeds begin to shrivel just before they are ready to disperse. Only occasionally do all four seeds ripen on one flower. Whether seed abortion is caused by resource limitation or inbreeding remains to be determined.

The means for seed set in both *H. venusta* samples were similar, indicating that bagging experiments did not change pollinator behavior and resulting seed set. *H. venusta* had a higher seed set than the non-rare *H. diffusa* var. *arida,* in this sample year, but the sample size for *H. diffusa* var. *arida* was very small and restricted to one population. Small populations are at risk of inbreeding depression, which could affect seed set and plant vigor. The larger a sample size is, the more likely it is that it will reflect the true population mean. Therefore it would be helpful to find a larger population of *H. diffusa* var. *arida* in the same habitat, if comparisons are to be made again. In 2004 I collected seed for germination experiments from a

larger, lower elevation shrub-steppe population of *H. diffusa* var. *arida* and my subjective impression was that seed set was lower than that of *H. venusta* in the same year, and seed abortion was common.

I don't believe that seed set in either species is particularly high, whether because of environmental conditions (resource limitation) or other factors such as pollen source and pollinator activity. The amount of seed abortion caused by inbreeding has yet to be assessed. Geitonogamy is likely quite high, based on observed pollinator movements. Seed abortion caused by inbreeding-avoidance would reflect an adaptive trait, eliminating less fit seeds and allocating resources to outcrossed embryos. If seed production in the non-rare species is consistently near 25%, then it should not be assumed that 25% seed set is, by itself, a cause of rarity in *H. venusta.*

It is important to continue to measure seed set and to add this to long-term records of weather, rainfall and pollinator counts in order to analyze variation and establish the upper and lower limits of what is normal for the species.

Seed germination rates and survivorship among seedlings would reflect the relative fitness of outcrossed versus selfed seeds. Unfortunately, there were problems with the hand pollination experiment (the lack of complete control over pollen movement), and the germination trials of seed from the field plants (flawed temperature control) and therefore germination data from this study does not conclusively demonstrate fitness in individual plants. In previous growing trials, it was observed that some clones did much better in pots than others (S. Reichard pers.comm. 2004), with the result that there were more of certain clones available in containers. Interestingly, seeds from some plants in the field study germinated at much higher percentages than others (Figure 1.5), and this is not correlated with percent seed set, or seed size. Seeds may have been affected by the damage, if any, that occurred when seeds dried out at the beginning of germination treatment, but it could represent an expression of the natural diversity in the genome.

CHAPTER II. POLLINATORS OF *Hackelia venusta*

Background

Pollen is the vehicle for gene flow between flowering plants. When pollen grains inside anthers mature, anthers dehisce (split open) to present it for collection or dispersal by biotic or abiotic agents. Abiotic agents, such as wind or water, disperse pollen more randomly than biotic agents (insects, birds or other animals) that often visit individuals of the same species more than once, or sometimes only one species at a time.

In the process of collecting both pollen and nectar, insects deposit varying amounts of pollen on stigmas. If an insect is collecting only nectar, it may groom off almost all the pollen that sticks to its body before visiting another flower (Neff and Simpson 1997). They typically carry more pollen in hairs on their tongues and bodies than is transferred for pollination; they use it as food for their larvae and themselves, some falls off during grooming, or in flight, and some may not be on a body part that comes into contact with stigmas. Therefore the pollen that adheres to an insect *may* be available for deposition on a stigma, but not even all pollen in a position to be left on a stigma is deposited at the next stop. The time the insect spends in a flower, the way it handles the flower parts, and different grooming behavior with different pollen species can affect the rate of deposition (Robertson 1992, Rademaker and De Jong 1998, Mitchell et al. 2004).

The study of pollinator movement has been important in refining our understanding of population structure in plants. Robertson (1992) collected stigmas, and measured pollen deposition to estimate *pollen carryover* – the fraction of pollen that remains on the body of an insect after a stop to gather more nectar or pollen. He argued that carryover reduced self-pollination (geitonogamy) by holding some pollen in reserve until it is deposited on a neighboring plant. Powdered dyes are sometimes used as pollen analogs to estimate the amount and rate of pollen deposition from the original source (Rademaker and De Jong, 1998). Insects pick up the powder and

leave fractions on flowers as they move between plants. If the dye is close to the same weight and stickiness as the pollen being studied, actual pollen movement can be approximated, but this is not as precise as counting the real pollen grains on stigmas and insects.

Intuitively, it might seem that many flowers blooming simultaneously on one plant would attract more pollinators, and be an advantage to the plant. But the trade-off in this strategy is that there can be more self-pollination (inbreeding) and less cross-pollination if a pollinator spends too much time on one large plant and less time going between plants or patches of plants. Harder and Barrett (1995) used genetic markers to show that higher rates of selfing in large floral displays were a "cost" to the plant known as pollen discounting (a lost opportunity for pollen to be used for cross pollination, which either reduces heterozygosity in self-compatible species, or prevents seed formation in self-incompatible species).

How hermaphroditic plants maintain viable outcrossed populations in these cases has interested researchers for some time. Harder and Barrett (1995) suggest that plants have avoided this problem by evolving mechanisms like protandry and/or protogyny, or by displaying fewer flowers over a longer blooming period, since the authors observed insects leaving few-flowered inflorescences sooner. Mitchell et al. (2004) found that although larger plants with more flowers in bloom received more visits, the *proportion* of available flowers pollinated was not greater in larger plants or displays, and in fact, "the proportion of available flowers probed decreased with display, resulting in nearly equal floral visitation rates…" Goulson et al. (1998) demonstrated that even though pollen transfer within plants increases with a larger number of flowers per plant, each flower in both large and small displays has about the same chance of being visited by a pollinator.

A number of interacting behaviors and plant biological processes make it difficult to predict actual rates of selfing and outcrossing. Harder and Barrett (1995) observed that bumblebee visits to inflorescences were influenced by the size of neighboring displays, and although pollinators may be initially attracted to larger

displays, their behavior after they arrive on the plants has been shown to differ among insect species working the same plants; even insect species within the same genus exhibit different flower- and pollen-handling behaviors (Mitchell et al. 2004). Notably, Mitchell et al. (2004) reported that in all treatments (of flower number per display) in their study of *Mimulus ringens*, the most common behavior was for bees to probe one flower only before leaving the plant, and greater than 30% of visits to the largest inflorescences in their experiment were in this category. Their genetic analysis showed a corresponding low number of selfed seeds.

If more than one species of pollinator visits a plant, the amount of pollination accomplished by each may be estimated by observing behavior, excluding them individually based on size, or by time of pollination (e.g. to observe the effect of night pollinators plants are bagged at night but not during the day). One can also collect pollen from captured insects, identify its source, and count or estimate the number of pollen grains on insect bodies or attached to stigmas immediately after a pollinator visits the flower.

I limited my investigation of pollinators to observing and collecting as many as I could in the short time available. This information should provide a foundation for further pollination ecology studies of *Hackelia venusta* that address more detailed questions of gene flow, population structure, and collection of additional species of pollinators.

Materials and Methods

In 2004 I visually surveyed the area designated the "main" population (Washington Natural Heritage Program 2004). I visited this site on four occasions during the day and once in the evening to assess plants in flower, scout for pollinating insects, check seed development, and to locate suitable plants for bagging the following year. In 2005 I made a total of 13 visits to the study area in subpopulations A, B and C from April 8 to July 24. Insect collections were made

between April 29 and May 27. I did not enter the main population the second year because it was too easily damaged by the foot traffic that would be needed to conduct even a limited bagging study. I spent one or two days in the field each visit. The subpopulations are also quite fragile, and would not be suitable for overnight stays or daily visits over the entire blooming season.

Field time in 2005 was divided between watching insect behavior, collecting insects, and checking and adjusting pollination bags over the three subpopulations. Collection opportunities were sometimes sacrificed to better observe pollinator behavior in the time available, and observations were "snapshot" samples over the entire blooming season rather than concentrated periods over several days to count and watch behavior in detail. I watched for insects in each part of the three subpopulations at different times throughout the day and attempted to collect throughout the day. The sun came over a ridge to the east around mid-morning, and insect activity was more likely between 10 am and 4-5 pm when the sun was not blocked by mountains and ridges. I observed an area for at least 10 minutes before moving to the next; sometimes staying up to 45 minutes if no insects appeared on *H.venusta*. A two-week rainy spell restricted insect activity during peak bloom in 2005.

I used a standard butterfly net for capturing bees and flies, and made a smaller one from mosquito netting that worked better near individual plants. I also captured insects by grabbing them gently by hand, attempting to capture only those I observed with their mouthparts on or in the corolla, or obviously gathering nectar or pollen. They were put in a killing jar and pinned for identification. After keying them to genus, I contacted experts for confirmation and identification to species.

To see if insects were attracted to particular colors, I put out colored bowls containing soapy water, in an open area with a lot of insect activity at peak bloom on a very warm (mid 80's F), still day. I also left sticky yellow cards (used to trap horticultural insects) out overnight at the beginning and again towards the end of bloom to catch night-flying insects.

Results

The following insects were collected in nets or by hand:

***Andrena nigrocaerulea* Cockerell (Hymenoptera)**
Coll date: 4/29/2005; 5/27/2005
Species det. by Terry Griswold
USDA-ARS Pollinating Insect-Biology, Management,
Systematics Research lab, Utah State University

***Protosmia rubifloris* Cockerell (Hymenoptera)**
Coll date: 4/29/2004
Species det. by Terry Griswold

Eulonchus possible species ***tristis* Loew (Diptera)** pending revision
Coll date: 4/29/2005; 5/19,20, 27/2005
Determined by Chris Borkent
PhD Candidate, Department of Natural Resource Sciences
McGill University, Macdonald Campus, Quebec, Canada

***Nicocles sp*. (Diptera: Asilidae) female**
Coll date: 4/29/2004
Det by Chris Borkent

Voucher specimens of these species are deposited with the M.T. James Entomological Collection, Washington State University. Specimens of the two Hymenoptera were also deposited with the USDA-ARS Pollinating Insect-Biology, Management, Systematics Research Lab, Utah State University, Logan, Utah.

A number of Thysanoptera (thrips) adults and larvae were collected in vials of alcohol throughout the 2004 season. These were not identified to species. I mounted one adult specimen on a slide for measurement. Miscellaneous smaller insects (collected in bowls and on cards), mites, and one caterpillar were not identified beyond Order.

Eulonchus, Andrena, and *Protosmia* were all captured after they had been observed with mouthparts on or in the corollas of *H. venusta*. *Nicocles* was on a flower but not seen feeding on nectar or pollen. *Eulonchus* tongues are long enough

to reach the bottom of the inside of the corolla without getting their heads into the corolla tube. Visits by *Eulonchus* to single flowers were relatively brief, with little movement other than inserting the proboscis into the flower, and they occasionally groomed after feeding. *Andrena,* by comparison, insert their whole head and thorax into fully open corollas, and move around inside. I did not have the opportunity to observe *Protosmia* feeding or collecting, but pollen adhered to the abdomens of specimens collected. I observed a small hovering insect near the flowers during late bloom in 2005 on two occasions but could not capture it.

I had seen thrips (Thysanoptera) in 2004 crawling in and on open flowers of almost every plant that I checked, and they were relatively easy to capture by knocking them from the flowers into a vial of alcohol. I observed thrips behavior while holding one on my finger. I also found thrips adults and larvae inside the flowers of potted plants. I looked for them in 2005, and there were a few in late April, but the numbers were much reduced and it was difficult to capture them. There were none on potted plants that had overwintered near the field location in 2004-05. After early season bloom 2005, I did not find any thrips but observed and collected red spider mites on several occasions inside and around the petals. There was no thrips damage to petals at any time in either 2004 or 2005.

In 2005 I collected one small caterpillar in some webbing. It had chewed some petals of one *H. venusta*, but in general it is very unusual to find any herbivory or disease on *H. venusta* and the petals are nearly always perfectly white and clean. The two exceptions to this in 2005 were one plant that had very distorted flowers – reduced in size with a peculiar chartreuse striping; and a number of flowers that had some browning from rain during peak bloom (especially if they had been bagged or if there were many flowering stems on a plant crowded together).

Colored bowls did not attract large insects; a few minute flies and other insects landed in the bowls. The insects captured on sticky cards were few and of a similarly small size, and they were never observed on *H. venusta* flowers. No large insects were captured at night, and I have never detected an odor from the flowers at

night or any other time. One *Diptera spp.* was found inside a mosquito net bag that contained one seed, and one parasitic wasp was found inside another mosquito net bag with no seed set.

Rain showers immediately past full bloom in 2005 made it difficult to work between May 12 and May 27, as the plants were too wet, and pollinators were sluggish or inactive. After inclement weather it was evident that seed set had been affected by lack of pollinator activity or by rain damage to the plants. Seeds were forming on flowering stems, but on many there was a gap with no green seeds but shriveled flowers, then more green seeds farther up the stem where later flowers were pollinated. The flower petals are very thin and easily damaged when they are wet and large plants with many blooming stems fall over and stick together when they get wet.

Discussion

Pollinators observed on and in *H. venusta* flowers are not rare, or specialists on this plant. Andrena *(Euandrena) nigrocaerulea* (Andrenidae) is a ground-nesting, solitary bee. The following information is from Michener (2000). Members of this genus construct a main burrow in the soil with cells at the end of lateral burrows radiating from it. In each cell, female bees deposit pollen provisions shaped into a sphere, and lay a single egg on top of the food mass. Cells are made at the rate of less than 1, to more than 1 per day depending on species and location. After hatching the larvae feed until pupation, the final stage of which is a hardier prepupa or defecated larva (referring to a pellet which is expelled at the onset of this final resting stage), capable of overwintering. *Andrena* may overwinter either as adults or larvae in the burrow. They emerge the following spring to mate and construct nests, which may exist near others, but are usually not communal. There is one generation per year.

The genus *Andrena* is common and widespread in temperate and xeric areas of North and South America. Laberge and Ribble (1975) show the distribution of *A. nigrocaerulea* throughout Washington, Oregon, Idaho and much of California,

Wyoming, and Nevada and parts of Utah and southern British Columbia. These authors list it as polylectic, and I occasionally noticed it stop briefly on *Penstemon* while working *H. venusta* flowers, but generally it seemed to be focused only on *H. venusta.* If the adults outlive the blooming season of *H. venusta* they may switch to another species as it becomes available. Special configurations of hairs (scopa) on the legs provide a site for pollen collection and transport (Michener 2000). There are hundreds of species in many subgenera of *Andrena*; only a small proportion have been studied in detail (Neff and Simpson 1997). Individual species vary in nesting habits, biology and morphology. Some have special hooked hairs on the legs or tongue for extracting pollen from small, enclosed corolla tubes, including species that specialize on plants in the Boraginaceae (Muller 1995), and it may be of interest to investigate whether *A. nigrocaerulea* posseses any special adaptation to Boraginaceae. Danforth (1989), and Neff and Simpson (1997) observed variations in the number of cells provisioned per day by members of Andrenidae, and the number of collecting trips it takes for a female to collect enough pollen for one cell with a single egg. This appears to depend on resources and perhaps weather, as well as the size and habits of the species. Danforth (1989) timed foraging trips and distinguished between pollen trips and nectar-only trips. Nectar was only collected on the last trip for a cell and it was used for sticking the pollen together to form it into a sphere, as well as food for the adult. This author also noted that some species take "days off" from foraging. This would explain why females lay and provision less than ten eggs in their lifetime (Michener 2000).

Protosmia rubifloris (Megachilidae) is the only member of the genus in North America. Its distribution is disjunct in the Mediterranean basin and western North America from British Columbia to Baja, Mexico. In North America there is another disjunct population in northern Arizona (Griswold 1986, Michener 2000). There is no indication that this species is rare in North America within its range. Its presence east of the Cascade crest may be a new reported location, since I did not find any reports of Washington state populations outside western Washington.

Michener (2000) mentions that bees that nest in wood or twigs may cross geographic barriers more easily because the nest itself can be transported by water or other means. *P. rubifloris* nests in pieces of wood or pine cones, and overwinters as an adult to emerge in early spring [Griswold (1986) reports this as unusual for *Protosmia*]. Multiple cells divided by thin partitions or empty cells are constructed linearly in a single burrow, using resin, and provisioned with pollen; usually males will be in outer cells and females in inner ones (Griswold 1986).

I did not have an opportunity to closely observe *P. rubifloris* collecting pollen or nectar. I collected two specimens in the main *H. venusta* population in 2004, and did not see any in 2005, although during late bloom, the small (relative to *Andrena and Eulonchus*), hovering bee or fly that I was not able to catch, may have been *P. rubifloris* or another species of hover fly. Pollen was present on the underside of the abdomens of the specimens I collected. Identification of pollen source was beyond the scope of this study.

Eulonchus is a genus in the family Acroceridae (Diptera) – a host-specific parasitoid that attacks spiders. Larvae in this genus are endoparasites of spiders in the suborder Mygalomorphae. Two subfamilies of mygalomorph**,** or trapdoor spiders, are parasitized by *Eulonchus* (Schlinger 1987). *Eulonchus* hosts are fossorial, or adapted for burrowing (Gullan and Cranston 2000). These spiders use their silk to line their burrows or make structures that help them catch prey, but it is not sticky. They are a primitive group that is also much more long-lived compared to more derived groups of spiders, thus the length of residence time of *Eulonchus* larvae in their bodies can be a number of years (American Arachnological Society 2006).

Schlinger (1987) reports the following life history information for *Eulonchus:* this species of parasitoid lays eggs on the ground (adults of other acrocerid genera lay eggs on vegetation or wood), larvae attach to the leg of a host spider, enter the body and travel to the book lungs. The larvae then enter diapause that may last years (free living larvae can only survive a few weeks). Before emergence for pupation the

final instar begins a period of destructive feeding that kills the spider host, but about 24 hours before feeding begins, the spider spins a web that is suitable for the *Eulonchus* larva to attach to for pupation after emergence. The specific nature of the association of this parasitoid and spider subfamily, and the fact that the dipteran parasite can induce this behavior suggests perhaps a long evolutionary association. Adults mate in spring and summer and lay up to 5000 eggs over a period of days. Adult *Eulonchus* are considered to be good flyers and pollinators. Cady et al. (1993) mention that these flies are not often seen but if present can be locally abundant (as they were in 2005 at the study site). One supposes emergence may be coordinated in some fashion for this to occur.

Nicocles is a member of a predatory genus commonly known as robber flies (family Asilidae). The individual I collected was probably waiting to prey on other insects arriving to feed on pollen and nectar, and is not considered a pollinator (C. Borkent, pers. comm.). Before it was captured, I observed an insect repeatedly and systematically buzzing around individual *H. venusta* plants, but not landing. Initially I thought it was assessing the nectar or pollen resources, but I believe this was the *Nicocles* looking for prey. Neff and Simpson (1997) reported an Asilid in one of their study populations of *Andrena*, but it captured a nest parasite of *Andrena rudbeckiae*, so the presence of a predator could be beneficial to some pollinators.

Of the very small insects observed or captured, thrips were by far the most numerous, but only in 2004. Thrips is the common name for insects in the Order Thysanoptera, and also the name of one genus in the family Thripidae (Bland and Jaques 1978). The Thysanoptera are ancient insects, existing since the early Permian 285-245 mya, (Gullan and Cranston 2000, ref in Mound and Terry 2001). About half the modern species are fungus feeders, and half feed on flowers, plant parts, and especially pollen. A few species can be found on moss and club mosses and some are predatory; even non-predatory thrips can be cannibalistic when nitrogen sources needed for reproduction are scarce (Kirk 1985, Mound and Teulon 1995). Thrips range in size from 0.5 mm to 15 mm. Their mouthparts are an asymmetrical

arrangement of a left mandible and two maxillary stylets enclosed in a feeding channel. The mandible punches into cells or pollen grains, and the contents are sucked out through the stylets. They can reproduce by parthenogenesis – a reproductive strategy to build up large populations early in the growing season Kirk 1984, Gullan and Cranston 2000).

Because many species are tiny, they are difficult to study (and to exclude from pollination experiments). Until recently they were mostly overlooked as important organisms in a biological context, unless they became pests. Studies of their biology have been mostly used for devising control measures (Kirk 1984a), although less than 1% of species worldwide are serious economic pests (Mound and Teulon 1995).

The scientific literature contains references to thrips in association with flowers since at least 1868. In 1876 Charles Darwin observed thrips moving between flowers carrying pollen on their bodies, and commented on them as a confounding factor in pollination experiments (ref in Kirk 1984). Still, for more than a century after that they were regarded as relatively unimportant pollinators of crops and wild plants, if at all (Kirk 1984, Endress 1994). They were noted as sole or important pollinators in only a few studies (Moog et al. 2002, Sakai 2002). Relatively recently, researchers have documented the effectiveness of thrips as pollinators of cycads and flowering plants in all parts of the world (Thien 1980, Baker and Cruden 1991, Luo and Li 1999, Mound and Terry 2001, Moog et al. 2002).

Because they are vulnerable to desiccation they will seek out crevices in flower parts which offer a warmer or cooler, more moist environment, and some protection from predators - though flowers are not necessarily havens from predation (Kirk 1984). Small flowers with constricted or obstructed corolla entrances, or buds that allow access to protandrous or protogynous flowers before corollas are fully open, are well suited to pollination by thrips. Winged thrips can orient themselves toward their host plants, hover, and navigate to the host preferentially based on scent and color. Moog et al (2002), and Mound and Terry (2001), observed directed flight

and purposeful behavior of large-bodied species in the field in Australia and Malaysia respectively; and Kirk (1984) conducted both laboratory and field experiments in England, with the same results. When they were abundant and easy to catch in the first year of my study, I was able to hold one on my finger and watch it walk back and forth, raising and lowering its abdomen, and launch itself into the wind - behavior that might have been an orientation maneuver.

H. venusta morphology fits the needs of thrips and other small insects very well. The larvae can be reared inside the corolla well protected in a warm, moist environment with a ready supply of nectar to feed on; and I found them in this happy living arrangement when hand pollinating plants. Although they may not be major pollinators, their abundance in the first year of my study, and the fact that they were not damaging the plants, led me to the conclusion that they could be secondary pollinators. The apparent population crash the following year may have been caused by weather, predators (e.g. predator mites), the end of a cycle of buildup, or a combination of these. Since there were so few the year that I used pollination bags, I did not get an idea of whether or how much they contribute to pollination, but they may contribute a measurable amount of pollination in years when their numbers are high enough. The bags used to exclude thrips were heavy and stiff, and tended to damaging stems more than lightweight mosquito net bags, so I would recommend either caging plants grown in a more protected, accessible environment in the field or conducting a thrips pollination study in containers in a facility as close to the field site as possible in years of high thrips activity. Monitoring by trapping and counting in subsequent years could be done without trying to measure pollination directly.

None of the large pollinators appeared in the bowls, either because the colors were not important, or because the insects were focused on food resources of their preferred plants. Sticky traps put out very near *Hackelia venusta* on two occasions and left for more than one week in 2005, trapped a small number of minute insects but no large insects.

The most probing and collecting by the larger insects seemed to occur in the morning, although further observation might indicate that there is more than one peak of activity, depending on pollinator. In the late afternoon on a very hot day, insects approached *H. venusta* and buzzed around more often than landing and collecting. Measurements of nectar production throughout the day, coupled with observations of collection activity might indicate that the supply of nectar is depleted later in the day, and there may a visual change in the flower that signals low nectar or pollen.

Pollinator Behavior and Effects on Pollination

It appears that the most important pollinators in 2004 and 2005 were the two bees (*A. nigrocaerulea* and *P. rubifloris*) and the dipteran spider parasitoid (*Eulonchus sp.*). These insects are the largest observed pollinators, and therefore probably move more pollen and move it farther than small insects, although a large number of thrips could potentially be quite effective. If the small bee or fly hovering around *H. venusta* flowers at the end of the 2005 season was *P. rubifloris*, its presence in both 2004 and 2005 might indicate that it is a regular visitor and major pollinator.

Eulonchus may not be consistently present from year to year because it depends on spiders for its development from egg to adult, and that period may last for more than one year. If this were true, its population would be more cyclical, and harder to predict. If the spider population changed, this would cause corresponding changes in the fly population. Adult *Eulonchus* do not need to feed their larvae with pollen so they are mostly nectar gathering, although adults may need to consume a certain amount of pollen to mature eggs. They insert their tongues without putting the rest of the head down inside *H. venusta* flowers, and in the process they probably pick up pollen since the anthers are immediately inside the narrow entrance and easily brushed by any movement. I saw them grooming from time to time, which I took to be pollen removal. They frequently visit several flowers on a plant, move to a

neighboring plant in a grouping, and then revisit previous plants, in apparently random fashion. They are therefore likely to affect geitonogamous selfing as well as outcrossing. Their abundance might make up for the smaller quantity of pollen handled, if only a few grains are necessary for pollination and they can move it throughout the population and among many plants. Furthermore, since they move more quickly between plants, they may be pollinating more total plants than insects that spend more time handling the flowers, but whether their role is a major or supporting one remains to be tested.

Andrena nigrocaerulea by contrast, actively probes flowers for a longer time, with its head and thorax thrust well inside the corolla. Its movements and time spent on each flower indicate that it may be gathering the most pollen, which is groomed off and carried in specialized hairs on its hind legs. The females are supplying their cells with pollen, and it takes other *Andrena* species several trips to provision one cell and egg (Neff and Simpson 1997). Likewise, *P. rubifloris* is collecting pollen for multiple cells in its nest, so it follows that these two species are working more systematically and may be moving the most pollen per individual at one time. *However, from the plant's point of view, it is unknown how this translates ultimately into pollination.* Measurements of pollen carryover and the number of plants visited would better demonstrate the contribution of each of these pollinators to outcrossing and selfing. It is important to establish a long term monitoring program directed at insects specifically so that populations can be counted more accurately and fluctuations in numbers and activity can be examined more closely.

Knowing how these insects relate to floral displays would fill in a major piece of the pollination picture. As mentioned above, Mitchell et al (2004) observed that an appreciable number (30%) of pollinator visits end after the insect probes a single flower. Additionally, it was mentioned that plants might have evolved a strategy of blooming over a long period with just one or two flowers open at one time to increase the chance of outcrossing (Harder and Barrett 1995). Both of these findings are relevant to *H. venusta.* Although "showy" *H. venusta* accomplishes this

by displaying a large number of stems with two, or at most three blooms open on each flowering stem each day. As the cyme uncurls, it continues to open one flower every day or so over a blooming period of about six weeks. Keeping in mind the studies mentioned in the introduction to this chapter, it seems possible that despite the architecture of the small, enclosed flowers, and a relatively small population, *H. venusta* could maintain outcrossing by presenting new flowers over a long period. *H. venusta* plants are scattered over their small range, in localized groups, perhaps because seeds fall to the ground and germinate close together. Insects were often seen going back and forth among neighboring plants before they left the area, so they might be attracted to larger clumps of flowering plants initially, rather than isolated individuals. Several smaller individuals with many stems in flower at one time might be just as attractive as a very large plant covered with blooms.

Monitoring and Long Term Study

In any given year it can be assumed that there will be variation in insect numbers and species, because insect populations are affected by many environmental conditions including predation, weather, competition, and host species or food plants. Future research should concentrate on developing study designs to assess pollinator diversity, abundance, and change over time and correlate the results with other environmental conditions if possible. Frankie et al. (2002) discuss study design for pollinator monitoring in more detail, including the type of flowers and patch sizes to choose, when and what to observe, and how to standardize methods for repeat data collection beyond qualitative presence/absence surveys. This last is important for collection of relevant data that can be compared from year to year. The authors outline methods that keep background variables constant, like time spent observing, time of day, and individual flower patches or plants, so that variation caused by environment or other species might be measured more accurately. Interestingly, they observed differences in the attractiveness to bees of individual plants within a population, which they suggest could be caused by intraspecific variation in floral

rewards. *Since H. venusta has been shown to be genetically diverse, and seed set, tissue culture success and germination has also varied between individuals, attractiveness to pollinators* is another layer of data that can help fill in details of survival and establishment that may have a genetic basis.

Frankie et al. (2002) also used trap nests as a tool to measure change in populations over time and bee diversity at different sites. They caution that, "They [trap nests] do not, however, provide insight on cause and effects of changed frequencies. It is thus important to plan ahead with specific complementary ecological studies that should provide information for interpreting bee frequency patterns. Obvious examples of this kind of study would include: i) studies on natural mortality factors; ii) monitoring usual and unusual weather patterns (e.g. drought periods, extreme rainy periods, El Niño and La Niña years); and iii) monitoring human disturbances (e.g. such as new or changing agricultural developments, pesticides, loss of preferred bee nesting habitats, fire, changes in local/regional hydrology)."

As the next step in monitoring, it would be very helpful to establish a yearly record-keeping system for temperature, rainfall, and soil moisture. Together with annual pollinator censuses and assessments of the general condition of plants, it might then be possible to find patterns of pollinator abundance or scarcity, and relate them to seed set on *H. venusta* in the context of environmental conditions.

Threats to Pollinators – Habitat

The life-history trait that the Hymenoptera and Dipteran pollinators share is the need for undisturbed nesting habitat. *Andrena* and the fossorial spider hosts of *Eulonchus* construct burrows in the ground, and *Eulonchus* females lay eggs on the ground near spiders. *Protosmia rubifloris* nests in wood or cones, and overwinters as adults, and so depends on the existence of some trees and undisturbed debris for a large part of the year. A primary goal of the Recovery Plan for *Hackelia venusta* (U.S. Fish and Wildlife Service 2007) is to maintain open, exposed habitat

conditions by treating the shrub and tree cover. This would be done manually or by conducting controlled burns. Both methods can be beneficial in renewing healthy growing conditions, but a stand-replacing event could have a detrimental effect on many resident invertebrates. The Xerces Society, a non-profit invertebrate conservation organization, has published guidelines for habitat management in natural areas that protect important pollinators and beneficial insects (Black et al. 2007). The recommendations for controlled burns state that only thirty percent of less of a site should be treated at any one time, refugia from treated patches should be available for invertebrates, and treatments should be done on a three to ten year rotation depending on site characteristics. Other recommendations about timing of treatments are contained in a fact sheet available on the Xerces Society website www.xerces.org.

Neff and Simpson (1997) found that *Andrena rudbeckiae,* which they describe as "robust" traveled up to 100 meters to forage. The species in *H. venusta* habitat may have similar, or smaller ranges. *Andrena* and *Protosmia* overwinter as eggs, prepupae, or adults. They mate soon after emergence in the spring and begin to feed. Therefore, it is very likely that their nest sites are within the plant population that they use for food. Spider hosts of *Eulonchus* live more than one year, and so would need stable and protected conditions throughout the year. The life history and physical location of all pollinators should be ascertained before modifying the habitat. The substrate in the *H. venusta* habitat is loose, gravelly, rocky and steep. In early spring there is usually enough moisture in the ground to hold the soil particles together. But as the season progresses and the soil dries out, it becomes extremely unstable and any foot traffic will cause local land slippage and damage. Disturbance from human activities can thus be very damaging to ground-dwelling pollinators and plants alike. Fortunately, the *H.venusta* population is mostly in a botanical research area. The site does not present any particular attractions for camping or viewing, and it is hoped that the site will continue to remain relatively intact.

CHAPTER III. GERMINATION PROTOCOL

Germination trials go hand in hand with seed production measurements, because we need to know whether seeds are viable. Looking at germination rates is one way to test the fitness of seed produced from different pollination events (i.e. selfing or outcrossing). A germination protocol is an important tool for those who work with endangered species, whether they are testing stored seed in seed banks, propagating them under controlled conditions for later outplanting, or broadcasting to increase natural populations. For biological information about a species to be complete, we need to know the environmental conditions required for germination. Manipulating embryos by excising them and germinating them outside the seed coat, or using plant hormones to stimulate germination can produce the maximum number of seedlings, but the researcher who is interested in plant populations will want to know how and when seeds germinate naturally (Baskin and Baskin 2001).

Germination testing of seeds should be performed soon after harvest, so that ecological information can be obtained about dormancy and environmental conditions required for germination. Testing seeds when fresh provides more accurate information because some seeds lose dormancy during storage through afterripening (Baskin and Baskin 2001). Seeds should also be removed from storage and tested periodically to monitor the effects of storage on germination and viability. Testing and record keeping are especially important if seed collections are intended for reintroduction or to replace small populations that may be extirpated or damaged. If apparently viable seeds do not germinate when provided with a favorable environment - a moist, warm substrate, for example - they are considered to be dormant (Baskin and Baskin 2001). This makes sense from an evolutionary perspective, because if dispersal occurs in summer or fall, even if sufficient moisture is present late in the growing season and temperatures are mild, it may be fatal for a seed to germinate if the plant cannot undergo necessary changes in internal tissues (hardening off) to survive a long period of cold weather.

Seed plants are genetically diverse, and they have colonized a variety of habitats on earth, so it is not surprising that the mechanisms of seed dormancy are not the same even in plants from the same habitat. Baskin and Baskin (2001) discuss six types of dormancy, including four subtypes of physiological dormancy. Even though the mechanisms of dormancy within seeds can be chemically and physiologically complex, the natural conditions that promote or break dormancy are limited to environmental variables: temperature, moisture, or heat from wildland fire, for example. Treatment of seeds with either warm or cold stratification can induce germination in all types of physiological dormancy, and in two other types of dormancy as well. A "move-along" (Baskin and Baskin 2003) experimental technique allows the researcher to test more than one type of dormancy at one time, and to set up experiments with controls and treatments to generate data using the fewest seeds possible (Baskin and Baskin 2004).

Although each species should be tested separately to confirm the exact requirements of each, closely related species of the same genera might be assumed to have essentially the same requirements for germination. To further minimize the use of *H. venusta* seed for experiments, I used seed of *Hackelia diffusa* var. *arida* (Diffuse Stickseed) as a surrogate to work out a seed germination protocol. Genetic analysis by Hipkins et al (2003) showed this species to be the most closely related to *H. venusta* among several members of the genus. I then germinated the seeds from hand pollinated potted *H. venusta* clones, and finally wild collected *H. venusta* seeds using the protocol that yielded the best germination for *H. diffusa* var. *arida.*

Materials and Methods

Facilities available at the time I tested seeds of *H. diffusa* var. *arida* and hand-pollinated *H. venusta* were a greenhouse, growth chamber, and walk-in cooler. Later, incubators with controlled temperature and lighting were available for germination tests and these were used for *H. venusta* seeds collected from the field study.

H. diffusa var. *arida* Germination Test

I collected mature, brown, dry seeds as they were dispersing from twenty plants on June 9, 2004. The seeds were placed in the Miller Seed Vault at the University of Washington Center for Urban Horticulture. Although is best to test seeds immediately after collection, they were stored at low temperature and humidity for the next few weeks. Lowering temperature and humidity for storage prolongs the storage life of seeds, and slows the rate of change that might be occurring due to seed metabolism.

I tested two groups of 20 seeds each for seed coat permeability. One group had been warmed to room temperature. The second group was used directly from the seed vault (15 ° C). I weighed each group of seeds before placing them between two sheets of damp filter paper in separate Petri dishes. I weighed each dish again at1 hr, 2 ¼ hr, 8 hr and 22 ½ hr intervals.

From July 13 to August 6 I tested three groups of 35-39 seeds for dormancy by placing them on moist filter paper and exposing them to a range temperatures. I placed a container outdoors (air temperature approx 27°C day/ 17 C night), indoors at room temperature (approximately 26°C day/18°C night) and in a cool basement at a constant temperature (approximately 16°C). All seeds were exposed to diurnal light/dark.

"Move-along" technique (Figure 3.1). I set up three replicates of 50 seeds each in Petri dishes lined with moist filter paper in the greenhouse, growth chamber, and walk-in cooler as controls. These remained at spring, summer, and winter temperatures (fall is the same as spring) for the duration of the experiment. Two treatments (three dishes each with 50 seeds in each dish) were placed in the cooler and the greenhouse. These two treatments were moved at 13.5 weeks to the next seasonal simulation (winter to spring, and summer to fall, respectively). The greenhouse was heated and had some supplemental lighting during late summer and fall, and I maintained 29-30°C daytime temperature by putting the dishes on a propagation mat with a thermostat. On sunny days, the greenhouse day temperature

rose higher than 38°C on occasion, and was frequently 32°C or above. Minimum temperature was 14°C +/- 2 degrees. It was easier to keep temperatures in the growth chamber (19°D/5°N) and cooler (3-4 constant) within set ranges. Lights were on in the growth chamber during the day period, and lights were turned on periodically in the cooler when seeds were removed, or when other users entered, but were not on a day/night cycle.

Using this configuration of treatments and controls, it was possible to test the effect of a warm period followed by cold (the treatment started at summer temperature and moved to cold) or cold alone (the treatment started at the winter temperature) in breaking dormancy. The effects of long exposure to one temperature regime could also be tested.

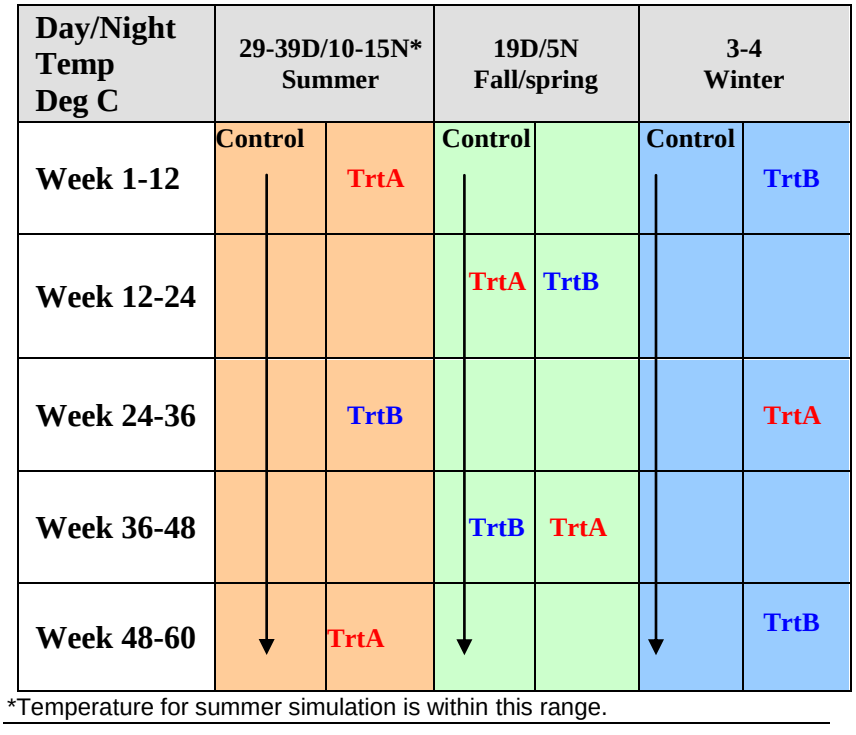

Figure 3.1 "Move-along" germination treatments (after Baskin and Baskin 2003)

H. venusta – Seeds From Hand Pollinated Plants

Seeds collected from hand pollinated *H. venusta* were placed in the UW Miller Seed Vault as they ripened. Seeds from each clone were separated by parent plant and labeled with type of pollination: s = selfed/geitonogamous; ss = selfed/autogamous x = cross pollinated with different clone; control/bagged = bags over pots, not manipulated. On December 9, 2004 all seeds were placed in Petri dishes lined with moist filter paper and incubated in a walk-in cooler at 4-5°C. There was no scheduled lighting, but lights were turned on when the door was opened periodically. I did not use a full move-along experiment because the results from the previous trial with *H. diffusa* var. *arida* indicated that cold stratification would break seed dormancy.

H. venusta – Field Collection

Seeds from each pollination treatment and from an additional open pollinated control stem were collected in 2005 from the field study. (Untreated stems were not randomly chosen because they had to be strong enough to support a seed collection bag.) Seeds were collected when fully ripe and dry, separated by parent plant and pollination treatment**.**

Incubators were then available for germination testing, and seeds were placed in Petri dishes on moist filter paper in a Percival Model I-30BLL environmental controller at 5°C day/2°C N with 10 hours of light. All seeds were placed at the same temperature since cold stratification had been most successful in the previous two trials.

Seeds had to be moved from the incubator two weeks later, and were placed in another incubator of the same model. Unfortunately an error was made in programming the second incubator, and the temperature setting was incorrect. The seeds warmed up to approximately 23°+ C and dried out over a period of approximately ten days. When the error was discovered, the seeds were re-imbibed and replaced at the lower winter temperature and short day regime. When

germination was below expected, based on previous germination trials with *H. diffusa* var. *arida* and hand pollinated *H. venusta*, I decided to begin a rotation of the field collected seeds through the seasonal simulations. This was based on my professional propagation experience when I had been able to increase germination in other species by exposing them to alternating warm and cold cycles. Therefore, I rotated all seeds on the following schedule for the remainder of the experiment:

Table 3.1 Seed treatment – wild collected *H. venusta* in incubators

Temperature Deg C	Weeks of Treatment
Cold (5°D/2°N) 10 hrs light	22
Cool (15.9°D/7.9°N) 12 hrs light)	7.5
Warm (23.4° D/14.1°N) 14 hrs light)	8
Cold	21
Cool	10.5
Warm	8
Cool	8
Cold	41

As seeds germinated, they were removed and planted in 2" plastic pots in Sunshine Mix #2 (Manufacturer: Sun Gro Horticulture Canada Ltd.). The potting mix contains Canadian sphagnum peat moss, coarse grade perlite, gypsum, dolomitic lime, and a wetting agent. Seedlings in pots were placed back in the cold winter temperature incubator. More details on transplanting and growing seedlings will be found in Appendix A.

Results

H. diffusa var. *arida*

Imbibition. Dry seeds placed on moist paper absorbed water, as measured by increasing weight. Since seeds must imbibe water to germinate, this test for seed coat permeability indicates that the seed coat is not preventing water uptake (i.e. seeds do

not possess physical dormancy) and the seeds can be tested for other types of dormancy.

Germination test. Seeds did not germinate after imbibing water when placed at a variety of temperatures for three weeks, and were judged to be dormant. A new group of seeds were tested using the Baskin and Baskin (2003) move-along protocol. *H. diffusa* var. *arida* treatment and controls began to germinate in the cooler on approximately October 8, after nine weeks of cold stratification. Typically, on healthy seedlings the radicle emerged first, from the ridged side of the seed attached to the plant. The outward-facing "front" of the seed is flat with outward facing prickles (Figure 3.2). Occasionally the cotyledons emerged from the seed coat first. Seeds germinating at cold temperatures are generally healthier, probably because they are less likely to be attacked by fungus.

By the end of the twelfth week, 37.3% of all seeds (treatment and controls) in cold stratification had germinated. Seeds continued to germinate in cold at a slower rate until the end of the twenty-third week, and thereafter sporadically until the thirty-fourth week when the experiment was terminated. The treatment group was moved from the cooler to the growth chamber (spring temperatures) after thirteen weeks, and germination slowed down for that group in the warmer temperatures, with the last recorded germination at the end of twenty-six weeks. Four seeds germinated after the move to warmer temperatures. One seed germinated after moving the treatment to very warm summer temperatures, during week thirty-three, but it was not a robust seedling.

Figure 3.2 *H. diffusa* var. *arida* seed germinating *J. Taylor*

The germination pattern between the control and treatment groups was slightly different, with a higher number of treated seeds germinating sooner, especially during the twelfth week (Table 3.2; Figure 3.3). At the thirteenth week, when the treatment group was moved to warmer temperatures, 60 seeds (40% of the treatment group) had germinated in the treatments, representing 92.3% of total germination in the group, compared to 52 controls (34.6%) representing 73.2% of total germination. This difference in germination rate was not significant ($P = 0.696$) and assumed to be within normal variation at $\propto 0.05$ confidence level.

The control group kept in the cooler continued to germinate at a slower rate after the initial peak at ten weeks and eventually surpassed germination in treatments moved to warmer temperatures. After 34 weeks, total germination for controls was 71 seeds (47.3%) compared to 65 (43.3%) for the treatment group. There was no significant difference between the mean final germination percentages (Paired two-sample t-test or means $t=-0.30$, $df=14$, $P= 0.77$).

Table 3.2 Germination percentages for cold treated seeds of *H. diffusa* var. *arida*

Week	# Seeds Germinated		% Germination (cumulative)		
	Controls (cold)	**Treatment (cold + cool + warm)**	**Controls (150 seeds)**	**Treatment (150 seeds)**	**Treatment and Controls (300 seeds)**
10	29	30	19.33	20.00	19.67
11	4	3	22.00	22.00	22.00
12	8	23	27.33	37.33	32.33
13	11	4	34.67	40.00	37.33
14	3	1	36.67	40.67	38.67
15	3	2	38.67	42.00	41.00
16	2		40.00	42.00	41.00
17	3		42.00	42.00	42.00
18	2		43.33	42.00	42.67
19	1		44.00	42.00	43.00
20	1		44.67	42.00	43.33
22	1		45.33	42.00	43.67
24	1		46.00	42.00	44.00
26		1	46.00	42.67	44.33
33	2	1	47.33	43.33	45.33
Total	**71**	**65**	**47.33**	**43.33**	**45.33**

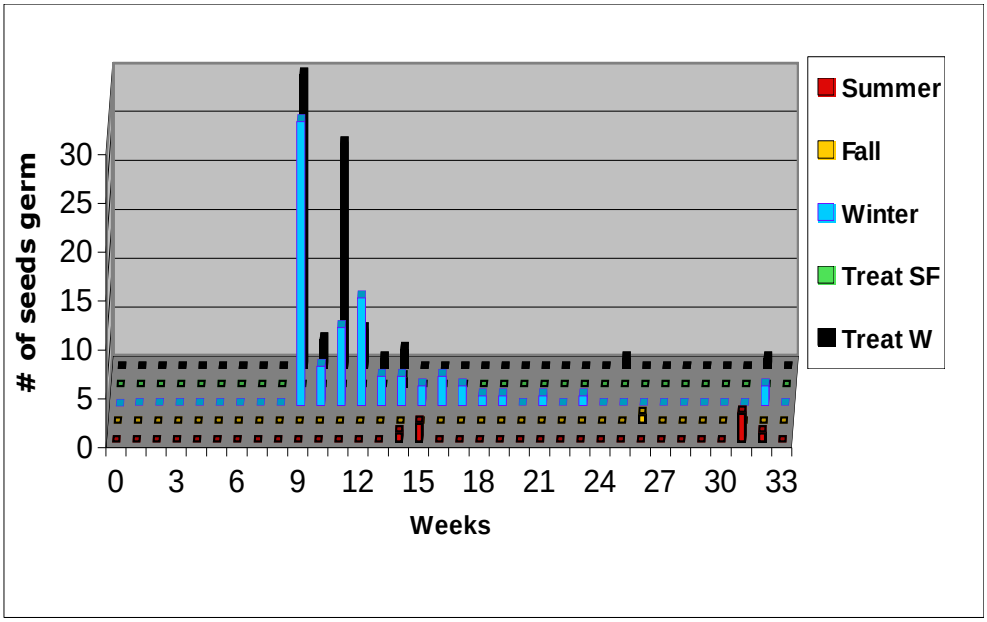

Figure 3.3 Germination of *H. diffusa* var. *arida* after cold treatment

Controls kept at warm (summer) and cool (spring/fall) temperature, and treatments that began at the summer temperature regime and passed through cool and cold treatments did not germinate well. One seed from summer controls germinated during the 14th week, two during the 15th week, three at thirty-one weeks, and one after thirty-two weeks of continuous warm temperatures. Germination percentage for the summer control group was 4.7%. One seed (0.7%) from the cool spring/fall controls germinated during the twenty-sixth week. One seed (0.7%) germinated in the treatment moved from summer to fall, during the second week in cooler fall temperatures. None of these seedlings were robust in comparison to cold stratified seeds and were sometimes discolored at the radicle or on the stem.

H. venusta, Hand Pollinated

Forty-five seeds were harvested from hand pollinated plants. Seeds began to germinate in cold stratification on March 11, 2005 after 13 weeks of cold. The last germination recorded was on May 27 at 24 weeks when the experiment was

terminated. The largest number of seeds (9) germinated between week 15 and 17 (Table 3.3), and final germination was 42.2%.

Table 3.3 Germination rate for seeds from hand pollinated *Hackelia venusta*

Week #	crossed (x)	selfed (s)	selfed (ss)	control/ bagged
13	3			
15	2		1	
16	3		1	1
17				2
21	1			2
24		3		
Total	9	3	2	5
% Germ	75	42.9	100	20.8

?= unknown; control/bagged no manipulation; s=selfed (geitonomgamous; ss= selfed (autogamous); x=crossed

H. venusta, Field Collection

Field collected seeds began to germinate on December 22, 2005, 19.5 weeks after the beginning of the experiment and 14.5 weeks after the second imbibition and restart of cold treatment. Only two seeds germinated on December 22, and three on January 12, 2006. All were from open pollinated stems. During cycles of simulated seasonal warming and cooling, germination spiked after each cold period (Figure 3.4).

At 140 weeks the total germination percentage from the beginning of the experiment was 14.12% for all seeds, 14.57% for the subset of open pollinated seeds, 9.09 % for seeds from stems with mosquito netting, and 6.67% from stems with thrips netting. There were still seeds from several plants that had very little fungal growth on the filter paper or around the seeds, and the seeds themselves were firm and plump and apparently still viable, yet ungerminated. Table 3.4 shows

germination totals and mean per plant germination by treatment taking into account those plants that set seed.

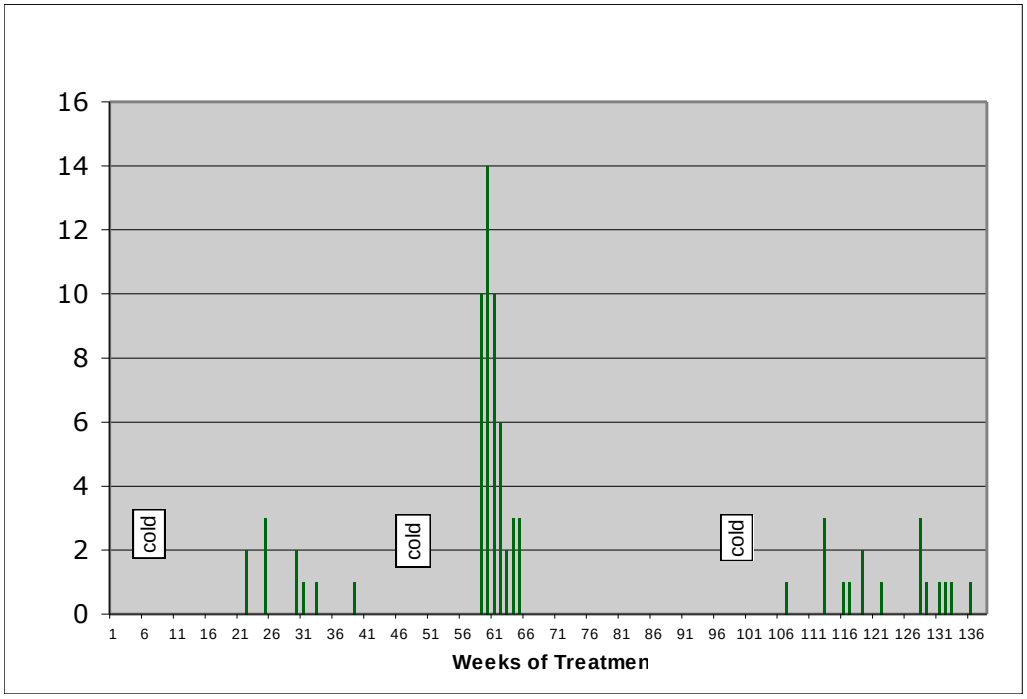

Figure 3.4 *H. venusta* germination over 140 weeks

Table 3.4 ***Hackelia venusta*** **wild collected seed germination**

Plant #	Seeds Collected			Seeds Germinated			Mean per plant germination % for plants with seeds		
	Ctrl	M	T	Ctrl	M	T	Ctrl	M	T
1	8	4		0	1		0	25	
2	52	1	0	4			7.69	0	
3	10		9			1	0.00		11.11
4	81	7	4	4			4.94	0	0
5	59	0		37			62.7	0	
6	85		2	15			17.65		0
7	68	1	0	2			4.41	0	
8	6			2			33.33		
9	37	4		0			0	0	
10									
11	17	0	0	2			11.76	0	
12		1						0	
13	17	3	0	5	1		23.53	33.33	
14	54	1	0				0.00	0	
Unknown	1			1					
	494	22	15	72	2	1	13.84%*	7.29%	3.70%
Total germination all plants and treatments (75/531) = 14.12%									

Ctrl – control stem
M – mosquito net
T – Thrips net

Blank - plant died/bag lost

*total open pollinated germ incl unkn = 14.57%

Discussion

Fresh seeds of *H. diffusa* var. *arida* did not germinate when imbibed without treatment. Seed coats were permeable to moisture, which indicated that there was no physical barrier in the seed coat preventing germination. Germination testing using a move-along experiment (Baskin and Baskin 2003) brought seeds out of dormancy with cold moist stratification. This treatment also broke seed dormancy of seeds from hand pollinated *H. venusta* tissue-cultured clones, and the time to germination and percent germination was roughly the same for the two groups. Germination for wild-

collected seeds from the population was much lower, but drying out after imbibition likely negatively affected the germination rate for these seeds.

These experiments established that seeds of both species exhibit non-deep physiological dormancy (Baskin and Baskin 2001). It is important to emphasize that the seed coat does not need to be physically nicked or abraded to allow water uptake. It is sometimes assumed that if seeds do not germinate it is acceptable to scarify the seed coat to induce germination, and although some seeds may have both an impermeable seed coat (physical dormancy) and require cold or warm stratification (physiological dormancy) this is not the case when seeds imbibe water. Seed size does not appear to be correlated with seed germination. Some very small seeds germinated earlier than large ones.

The length of warm and cold treatments for wild-collected *H.venusta* seeds differed from the original protocol. In the interest of time, and because I was uncertain about future germination, I shortened the usual 12-13 spring and summer seasons to approximately eight weeks, skipping a cool fall period in order to begin the third cold stratification sooner. I moved seeds in cold treatment to a warmer temperature when germination had slowed to one seed every two to three weeks, hoping to get the maximum number to germinate. In the natural environment, based on temperature and amount of daily temperature difference, cool fall temperatures last about two months, the coldest winter period about four to four and one-half months, spring two to two and one-half months, and summer three to three and one-half months (Western Regional Climate Center 2008). Although it appears that the length of cold stratification is the most important trigger for germination, it is unknown what internal changes the length of time in warmer temperatures causes. Since seeds were induced to come out of dormancy a second and third time (discussed below) exposure to warm temperature does apparently affect later germination cycles.

Species-specific response to cold. There was a difference in the length of cold required for *H. venusta* and *H. diffusa* var. *arida* to germinate. *H. diffusa* var. arida

began to germinate after nine weeks of cold. *H. venusta* seeds from hand pollinated clones and wild collected seeds germinated after a thirteen-week cold period. *H. venusta* seeds from the field consistently required thirteen weeks of cold, more or less, to germinate even after reentering dormancy. These responses to cold illustrate the need for separate testing of each species. It would be interesting to test seeds of *H. diffusa* var. *arida* from the same elevation and habitat as *H. venusta* to see if habitat had an effect on the length of cold stratification needed to break dormancy.

Dormancy cycles. The temperature error in trials for *H. venusta* seeds from the field may have been responsible for the much lower germination rate of this group of seeds. However it provided an opportunity to examine the effect of repeated cycles of cold and warm treatments and brings up some interesting questions.

The germination results show that *H. venusta* seeds can survive periods of drying and/or warming (seeds were kept moist during warm treatment, but did dry out occasionally) by moving between dormancy and nondormancy. Baskin and Baskin (2001) discuss this attribute of some perennials that germinate in spring or late winter after a cold period. Some species' seeds are triggered into a fully dormant state (they do not germinate at any temperature) by a warm and dry summer season, but will germinate when re-exposed to cold moist stratification. This may indicate that a seed bank exists in the natural habitat that can remain viable for several years.

When raising plants for reintroduction, it is important to recognize the need for several cycles of warm and cold to germinate all viable seeds. This would help preserve genetic diversity in populations raised *ex situ* for reintroduction. That germination occurred after imbibed seeds were warmed up and dried may also indicate that seeds are able to withstand a certain amount of changeable weather conditions. Just how many cycles of warm and cold stratification would be needed to germinate all seeds that are viable should be the subject of further investigation.

A few seeds germinated during either cooler spring-like or warm summer temperatures and may indicate that there are some seeds in a state of "conditional" dormancy (Baskin and Baskin 2001) that will germinate within a restricted

temperature range, but the seeds that germinated soon after the move to a new temperature could also be responding to the previous (usually cold) temperature. Since the summer environment of both *Hackelia* species is mostly dry, it would make sense to try drying them completely during the warmest treatment. After the fourth cold treatment the germination rate was lower. I wondered if some seeds have been sent into an especially deep dormancy by the original warming and drying, and I decided to leave seeds in cold treatment longer to see if they could be induced to come out of dormancy with an extended cold period. Although seeds did continue to germinate, the response was slow. Baskin and Baskin (2001) mention that even though germination can be induced after seeds return to a dormant state, some seeds may germinate to a lower percentage after they undergo repeated transition from dormancy to non-dormancy.

Since conditions for germination of wild-collected seed did not match those of the first two trials with the surrogate species and hand-pollinated clones, a trial with fresh seeds from the field population should be repeated. Even though there will be a temptation to avoid using seeds of the endangered population for testing, it is important to confirm that low germination is *not* a reason for its rarity. The total germination rate for all treatments combined for hand pollinated seeds, and for open pollinated *H. diffusa* var. *arida* was about 40%. If lower germination were obtained with temperature and moisture well controlled, it would indicate that the wild population does have different characteristics from the surrogate species and from clones that were used in hand pollination.

Table 3.4 illustrates a difference in seed germination rates and timing among individuals in the field. Only one group of seeds – plant #6 – germinated at least one seed during each full cold period. Although these results may be clouded by the temperature control error, individual variation seems to be an important life history trait that may have a large effect on population structure.

The final picture that emerges from germination trials of the surrogate and rare species is that relatively good germination can be obtained after sufficient cold,

moist stratification. The field population may be highly variable or more affected by amount and type of pollination. In future trials it would be important to keep track of individual lines, and to examine whether pollination activity is affecting germination rate.

CHAPTER IV. CONSERVATION RECOMMENDATIONS For *Hackelia venusta*

The Recovery Plan for *Hackelia venusta* (U.S. Fish and Wildlife Service 2007) predicts a need for augmentation of the existing population unless new occurrences are found within the current range of the species. Numerous attempts to locate new populations have been unsuccessful (T. Thomas pers. comm. 2008). The following recommendations for conservation and augmentation of the population are based on my research, observations, and horticultural experience.

A Washington Natural Heritage Program report (Gamon et al. 1997) mentions an observation from 1984 of a "high rate of seed abortion" (estimated at 60% to 70%) in the main *Hackelia venusta* population. It is difficult to determine what a normal range of seed production is for a species with only one known population, and no previous baseline data. However, since it has been called out as a possible reason for population decline (U.S. Fish and Wildlife Service 2007), this aspect of *H. venusta* biology needs to be examined more closely. Some steps to approach this question are to 1) assess current rates of seed production and compare these with similar species in the family or genus. My data from one season comparing seed counts from *H. venusta* and *H. diffusa* var. *arida* were intended to begin this process; 2) count seed from a sample of the population at regular intervals; 3) coordinate seed counts with pollinator monitoring using *consistent, replicated methodologies;* and 4) support these data with equally rigorous observations of plant establishment and survivorship as it relates to previous years' seed production and germination rates. Admittedly, for this species in its natural habitat, this will be a very difficult task because plants are easily damaged in the process of studying them.

Based on my measurements and a literature search, seed production of *H. venusta* is within the range of other members of the Boraginaceae, and higher than a small sample of the non-rare *H. diffusa* var. *arida*. Pollinators are active, not endangered, or specialists on *H. venusta*. Germination rates for field collected seed

were less than that of the non-rare *H. diffusa* var. *arida,* and hand pollinated *H. venusta* clones, but seeds may have been damaged when they warmed up and dried out soon after the beginning of cold treatment. A sample of at least 50 (preferably 100) seeds from throughout the existing population - including plants at the highest, mid, and low altitude sectors - should be retested for germination. If this sample germinates at a rate of 35% to 40%, it can be assumed to be near the baseline established by my experiments. If germination is much lower, then studies should be undertaken to determine the reasons for the lower germination. Work could be started to augment the population while looking into germination rates.

We still need to find out where seeds and plants do best. It is unclear why plants grow in some places and not others that appear to be equally suitable. Substrate seems to be the primary factor affecting establishment and health of individual plants, but other, less obvious environmental conditions, such as amount of groundwater, shade, leaf temperature, and adaptation to land slippage, slope, and aspect, seem also to be important. It is difficult to track either plants or seeds in the field, because of slope creep.

Two alternatives to increase the number of plants are to either place seeds on the site to germinate naturally, or raise seedlings offsite for outplanting. There are advantages and disadvantages to each method (Table 4.1). Seeds that germinate onsite have a better chance of getting roots down quickly and establishing before the soil dries out. The survivors are more likely to be the most fit, whereas seedlings raised offsite and able to survive pot culture may or may not be well adapted. Seedlings brought to the site and replanted will need to recover from transplanting. In most horticultural situations, this usually takes a few weeks if temperature and moisture are adequate, and transplants can catch up with plants growing onsite during the season if they are not stressed by environmental conditions. In a field situation, it is difficult to predict how transplants will perform. The extra money and time to germinate seeds and raise plants may be wasted if they do not establish. However, if the season is unusually dry, seedlings could be held until soil moisture is

more favorable, whereas seeds outdoors only germinate after the cold, moist conditions of winter and early spring. If seeds do not germinate the first year, germination could be lowered after the second winter chilling period (see discussion of cycling in and out of dormancy, Chapter III).

Table 4.1 Comparison of methods for increasing plants onsite

SEED PLACEMENT	SEEDLING INTRODUCTION
Natural germination and survivorship	Precise placement
Early establishment (stronger seedlings?)	Could wait for favorable moisture
Harder to track plants?	Easier to identify individual plants?
No guarantee of a moist spring	Transplant shock?
Potential damage/loss of seed	Waste of plants/narrowing of genome if unfit plants introduced?

Perhaps a third hybrid method to disseminate extra plants would be to sprout seeds in a laboratory setting using the germination protocol (13 weeks of moist cold) then place the newly germinated seeds in the environment immediately. This method only works if seed germination can be timed to coincide with favorable environmental conditions and access to the site (e.g. air temperature above freezing, and snow not too deep). Placement at the site would need to be in late winter when seeds are naturally beginning to germinate, and when ground water is at a maximum.

Whether for direct seeding or raising seedlings, a genetically representative collection of seeds from throughout the population should be used for augmentation so that individual genetic lines are not over- or underrepresented. It is best to place these in the existing population. (A small population of *H. venusta* founded on tissue cultured plants from a few genetic lines was introduced about ten years ago, to a location not far from the original population. About ten percent of the original plants survive [S. Reichard pers. comm. 2008]. This is encouraging, and this site should be

examined to document the conditions that promote successful establishment, but augmentation in the existing population is a more efficient way to leverage natural pollination and genetic variation, and use the limited amount of seed to best advantage).

Since we don't know whether placing seeds or plants will be more successful, techniques need to be empirically developed through a process of educated trial and error. Efficient use of resources requires that responsible parties make a long-term commitment to monitor results and collect data into the future. Unfortunately, time and money are often in short supply for conservation efforts, and this case is no exception. If a project cannot be sustained over the long term, it would be better to either scale back expectations for recovery, or try to advance toward the goal incrementally. For example, an overly large outplanting or seed placement event could create more sites than could be revisited each year. If the sites could not be monitored often enough, land managers could lose track of plants and not be able to gauge the effectiveness of the effort. A project planned with discrete, self-contained stages could be implemented more gradually without losing ground, and managed on a flexible schedule if staff or funding is reduced.

First, an environmental monitoring program should be established to permanently record:

1) Temperature and rainfall.
2) Yearly surveys of pollinators should be made in early, mid and late bloom. If time is short, at a minimum record presence or absence, and identify insects to genus. Fluctuations in insect populations are normal; they can be caused by changes in weather, food resources, disease, predation, and disturbance to nesting sites from fire or trampling. In a protected natural area, pesticides may not be an obvious influence, but since commercial agriculture is part of the economy in this part of the state, distant effects on pollinators should not be overlooked. If pollinator populations are not monitored on a regular basis, it will be impossible to

discern patterns of change over time, and thus learn how the pollinator-plant relationship affects both partners.

Frankie et al. (2002) emphasizes the need for clear guidelines and questions that are defined in advance when establishing monitoring programs. This includes planning for consistent data collection that can be replicated over the long term, and subjected to useful statistical analysis.

3) Seed set should be measured on plants from different elevations in the population. (See seed production note at the end of this section). Seed set percentages can only be calculated if flowers are counted at full bloom, and seed collection bags are added and gradually moved up the stem to catch them as they disperse. Early seed will be ripening while the late flowers are in bloom, so plants have to be visited repeatedly. Seed counts should be conducted every few years, if yearly counts are impossible, but they should be conducted as often as possible, and compared to weather and pollinator records.
4) Habitat management should be evaluated and coordinated with reintroduction. Measures taken to reduce tree and shrub cover and wildfire likely improved growing conditions for *H. venusta* in the past. for example, after the 1994 Hatchery Creek fire that passed through the habitat a small increase in the population was immediately apparent (U.S. Fish and Wildlife Service 2002). Fire ecology studies with management recommendations for this or comparable sites need to be acted upon in order to maintain favorable conditions, and support efforts to increase the population.

Keep in mind the effect that fire has on pollinators and plan accordingly. Prescribed fire can be useful for restoring a fire-adapted ecosystem, but it would destroy nests of *Protosmia rubifloris* in woody debris and cones at any time after the adults emerge in the spring.

Andrena may be better protected in ground nests, but even a fast moving, low intensity fire might kill larvae. At a minimum it would be prudent to prevent fuel buildup that increases the risk of hot, intense wildfires that heat deep soil layers, and move into tree crowns. Prescribed fires would be beneficial only to the extent that they were not conducted over the range of the entire *H. venusta* population at once, allowing pollinators to recolonize from unburned areas. Follow the Xerces society recommendations (Black et al. 2007) for pollinator conservation and consult with professional entomologists in advance on timing and amount of area to treat. *Pollinator monitoring programs will be very important* in determining the impact of fire or lack of fire on pollinators.

After the data collection, environmental monitoring, and habitat management plans are fully specified, work can begin on population expansion goals. Clear objectives to reach these goals should be developed as follows.

Objective #1: Establish a long term monitoring program for plants, pollinators, and weather. Establish data analysis and adaptive management procedures. Fund recordkeeping programs.

A long term monitoring program will permanently track the size and health of the population and its pollinators, and help to determine how these are correlated with weather patterns, habitat management, fire, etc. Managers should design research questions and data collection to incorporate unbiased statistical analysis. This guideline should describe methods and the timing for long term data collection.

Population-wide plant counts should be compared with data on individuals propagated through seed placement or plant reintroduction. Mangers should be able to follow the size and reproductive capability of the

study plants in subsequent years, and to learn something about plants as they age. Some questions to consider as the plants mature, include:

- Do they get larger each year?
- How long does it take for them to reach reproductive maturity?
- Does a large seed crop one year reduce the size of the plants the following year?
- Is there any way to estimate age of plants in the field based on some morphological characteristic?
- Is there a curve that defines the average lifespan, and what is the most productive period?

Objective #2: What introduction method yields the highest survival rate?

Experiment. Two unknowns are whether placing seed or individual plants is more successful, and whether spring or fall is a better time to introduce seedlings grown off site. The seed-versus-plant question can be addressed by placing groups of seeds and planting seedlings simultaneously, in an easily accessible location, for a paired test. This trial should be done in the fall, and again in the late winter (if resources are scarce, the fall and spring trials could be separated into two projects in different years). The scale would be small as a pilot study: ten seeds per group in ten groups, and five seedlings per group in ten groups. Pairing the samples would reduce uncertainty about the influence of weather from year to year. In all, four variations would be tested: fall seed planting, fall seedling introduction, spring seed planting, and spring seedling introduction. Seeds planted in the field in spring should be germinated with cold stratification in the lab to simulate being outdoors for the winter, but should not have shed their seed coat when placed onsite, i.e. radicle just emerging as in Figure 3.2),

To make the test more controllable and accessible, a site constructed either in the population, or at Forest Service headquarters would be more suitable for monitoring. Replicating the cliff-side and gravel slope in a more accessible location is the best way to raise plants. Stone-lined walkways would provide a way to monitor plants without trampling loose soil, and inserting plants into pockets within a rockery mimics the plants' natural tendency to grow in cliff crevices (a steep angle may promote good air circulation and drainage). Rocks act as mulch to keep the root run evenly moist and cool as the season dries and warms. If the test area is outside the population, rocks, soil and gravel from the field site should be used to mimic the slope, aspect and soil composition. If constructed in the field, it would be easier to lay paving to suitable sites near existing plants and mark the study plants and seeds. Special attention should be paid to providing adequate shade or limiting the hours of direct sun, and keeping the air temperature cool for some part of the day so that heat and moisture stress are limited. Keep in mind that small plants are less able to withstand extreme heat or cold, especially in an artificial environment where the soil may not be as deep or moist as in the field. The detailed instructions available in many books on alpine garden rockery construction offer the best advice on how to do this.
Evaluation. After gathering a year's worth or more of data from this trial experiment, there should be some information about whether seeds, plants, or both should be used to augment the population. If no plants survive from the trial introduction, the experiment should be restarted, with variations based on an adaptive management approach that reviews results from the first trial. Was moisture too limited? Were temperatures extreme? Are there ungerminated seeds that could still germinate after a second winter?

Objective #3: Plan seed collection, propagation, placement, and relocation of plant material. Plan a phased augmentation project. It is

important to collect seed from as many genetic lines as possible, in all parts of the population, including plants at high elevations and in cliffs when possible. Guidelines for calculating sustainable seed collection from rare populations (Menges, et al. 2004) should be followed carefully.

A map of the collection areas should be ready ahead of time, with staff and volunteers trained and available to take advantage of a large seed crop year (a large crop provides a larger sample, and the impact on the population will be less). Once seed is collected, the sample should be divided in two: one part for storage at the Miller Seed Vault at the University of Washington and the other for augmentation of the population. Seed vault stocks need to be replenished as they age, and since seed cannot be collected every year, it is most efficient to collect for all purposes at once.

Likewise, mapping locations for seed placement and/or plants ahead of time would make it possible to respond to changeable weather or site conditions without rushing through logistics. Finding seed caches or particular seeds that germinated at the end of winter after being carried by snowmelt or shifting substrate will be difficult. Seeds need to be in a container that allows moisture and air to penetrate, but is safe from animal damage. The container needs to be accessible for regular checks without undue damage to the site. When seeds germinate, the seedlings need to have room to get their roots into the soil be able to grow without being crowded by other seedlings or damaged by the container. Staff will need to brainstorm ideas for creative approaches to these problems (a biodegradable container? Velcro™ attached to a large boulder?).

A plan should be in place for funding facilities and personnel involved in propagation, and making sure there is space for plant storage in incubators until the proper season. Plants can be kept in cold or cool incubators for extended periods if necessary, so this might provide some flexibility in the project

Objective #4: Implement augmentation strategy. Staff should have funding and personnel available for collection, propagation and direct seeding. The quantity of plants or seed locations should be sized so that the staff available can monitor them indefinitely. The location of the sites for augmentation should be in the most stable and accessible areas, and managers should develop methods for increasing the stable areas available for growing *H. venusta*. One way to do this is to remove plant cover near existing plants, within the range of pollinators on those plants.

If the project has to be scaled back to just a small number of sites, then seeds or plant material from across the entire population should be grouped into those sites. Material from each plant should be tracked separately (perhaps by tagging the taproot itself near the crown). Continue data collection, analysis, and adaptive management of habitat and plants.

APPENDIX A. GROWING *Hackelia venusta*

The Draft Recovery Plan for *Hackelia venusta* (U.S. Fish and Wildlife Service 2007) predicts a need for augmentation of the existing population unless new occurrences are found within the current range of the species. It is hoped that the following recommendations based on my research, observations, and horticultural experience will support future reintroduction efforts.

Germination

Seeds should be germinated as soon after collection as possible. Stored seeds should be tested periodically, according to accepted seed banking protocols, to detect changes in viability over time in storage. Large differences exist in germinability and ease of culture between individual plants (this study, S. Reichard pers. comm. 2004); therefore it is important to keep records of germination by seed source and genetic lines.

Seed are dormant at maturity, and will begin to germinate after approximately thirteen to fifteen weeks of cold, moist stratification at 2 – 5 degrees C (see Chapter III). It is easier to study and handle seeds in petri dishes on filter paper, which should be moistened with deionized or filtered water. Incubators with good temperature control and lighting are ideal for maximum results. I did not treat seeds to prevent fungal growth, and have not found it necessary to do so for viable seed. Filter paper can be changed if algae or fungi begin to grow on the paper, but *H. venusta* seeds will tolerate a certain amount of fungal growth. Dishes should be checked often when seeds are imbibing water, to make sure the filter paper is moist, without standing water. Dishes may be enclosed in plastic bags when they are in cold stratification, if they cannot be checked every two weeks or so, but bags should be removed during warmer incubation temperatures. Light does not seem to be necessary for germination, but if seeds germinate and are not planted immediately,

lights will prevent excessive elongation of the stem. Light periods approximating the seasonal light/dark cycle should be used at each temperature.

Germination should be highest within one to two weeks after dormancy is broken, but seeds will continue to germinate at a slower rate if they remain in cold. Even though the protocol I followed called for exposing seeds to near-natural conditions of spring, summer, and fall temperature and light regimes for the length of time these seasons last in nature (twelve weeks is within a normal range) I adjusted these time periods to eight weeks for spring and fall, followed by thirteen weeks of cold, which saved some time. As noted in Chapter III, my first cold stratification period was interrupted soon after it started, so I consider the larger amount of germination after the second full cold period after restarting the experiment more typical, based on my results with the surrogate species *H. diffusa* var. *arida* and hand pollinated *H. venusta* tissue cultured plants.

To obtain the maximum number of seedlings, leaving seeds in cold for a longer period - two or more months after the first seed germinates - may be appropriate. However, the question that arises is, if seeds are rotated out of cold stratification after a prescribed "winter" chilling period, would the late-germinating seeds still germinate, either during the cool spring or after the next cold period? Statistically, the germination rate between *H. diffusa* var. *arida* left in cold and those rotated through warmer treatments was not significantly different.

After cold stratification, seeds should be *rotated through simulated seasonal temperatures, and cold stratified again*, repeating this yearly cycle for as long as seeds will germinate. Seeds will pass from a non-dormant to a dormant state as they are cooled and warmed, and each cold stratification period should yield further germination. Future research could establish the total number of warm/cold cycles needed to get all seeds to germinate that are capable of germination. Total germination percentages after subsequent cold periods may be lower, but high rates of germination may be observed for seed lots of individual plants, so it is important

to try to get all seeds to germinate. This investment of time is necessary to propagate and reintroduce as much of the genetic material as possible for this rare species.

Seeds will need to be watered more often in the warmer incubators. It is unclear whether allowing seeds to dry completely at the summer temperature is more productive than keeping them moist. This should be tested, perhaps with the surrogate species.

Handling seedlings

When seeds germinated, I planted them in 2" plastic pots in moist Sunshine Mix #2 (Manufacturer: Sun Gro Horticulture Canada Ltd.). I transplanted them as soon as the radicle emerged, handling them by the seed coat to avoid damaging the growing tip. I used a pencil tip to firm a few soil particles around the exposed root, keeping the seed coat on the surface of the thoroughly moistened potting mix. It is important not to bury the seed coat, but to have it rest in a small depression as it would naturally, near or on the surface, so that the emerging cotyledons will be immediately exposed to light. The roots then grow down between the soil particles and the seed coat falls aside as cotyledons expand and grow at their natural depth. In the cold incubator with pots within six to eight inches of fluorescent lights, stems stay compact and dark green, and grow naturally close to the soil. They can be grown in a cold incubator (2 – 5°C) for extended periods.

When roots fill the pots and seedlings have three or more sets of leaves, seedlings can be moved to an incubator with spring temperature and light settings. After more than six months in the cold incubator, seedlings in this study were transferred to a cool greenhouse. Perhaps because of temperature swings, humidity, fungus, or other factors, the plants did not do well in this environment, and were placed back in the cool incubator set to spring temperature and light levels, where they became much healthier (W. Gibble pers. comm.). Seedlings do best when they are grown cold or very cool. This may be a clue as to how to grow better plants in

containers for research, or if plants need to be held until they can be planted at the appropriate season outdoors.

Watering

It is important to keep soil moisture even. The temptation is to grow the plants on the dry side because they come from an arid climate. The leaves need to stay dry, but if roots dry out, plants will wilt very quickly. Seedlings in containers are not able to expand their root system to find water the way the do in nature – the volume inside the pot is all they have, and they do best when constantly, evenly moist but not saturated. No fertilizer was used until seedlings had been moved to a warmer temperature and began to grow more quickly. A weak (half-strength or less) all purpose houseplant fertilizer applied infrequently (this will depend on growth rate and how often plants need to be watered) should be sufficient to keep leaves healthy in the cool incubator. It is important to avoid salt buildup from fertilizer in the pots, so it is a good idea to allow the pots to dry enough so they can be flushed with water once in a while, allowing the extra water to run out the bottom of the pot and carry away salt buildup. The soil will be saturated and should be allowed to drain thoroughly. The proper soil mix will have large enough pore spaces so that after water drains, it will retain moisture and still allow the roots to have air – in other words all the pore spaces will not be filled with water. The pumice-to-peat ratio of Sunshine #2 provides this for small to medium sized pots.

Handling Mature Plants

Growing *H. venusta* in pots as mature plants has not been a great success. Plants have been kept alive for two to three years, but they are never as large as plants in the natural environment. They seem to be prone to winter damage, and vulnerable to drying out in the heat of summer if soil moisture in containers is not adequate. The Rare Care Program at the University of Washington found that overwintering container plants near the habitat was beneficial because of winter

chilling (S. Reichard pers. comm. 2004). I noticed that pots overwintered this way did not receive protection with mulch or insulation, and may have been damaged by an early freeze in Fall 2004. However they also did not survive well in the milder Seattle climate in 2005 or 2006.

The reason for their sensitivity may be that despite having what has been described as a taproot or a taproot split into longitudinal cords (Gentry and Carr 1976), an extensive network of very fine fibrous roots is attached to these thicker roots (Figure A.1). It seems probable that in natural conditions, the fine roots extend far from the crown of the plant toward moisture and nutrients, mining moisture deep in the soil, under large rocks, or in crevices of cliffs as the season warms and soil dries out. In pots, these fine roots may not be as well protected from cold, heat, and drying.

I preferred to transfer plants from plastic pots to clay pots because clay is not as dark and does not absorb so much heat. Clay also provides better air circulation. However, attention must be paid to keeping them watered regularly, because clay pots will dry out faster. It may not matter what material the pot is made of as long as the temperature swings are not too great and the roots are evenly moist. Pests were a problem outside of the natural habitat, especially aphids and slugs. This should not be so much of a concern if they are raised nearer to their natural range.

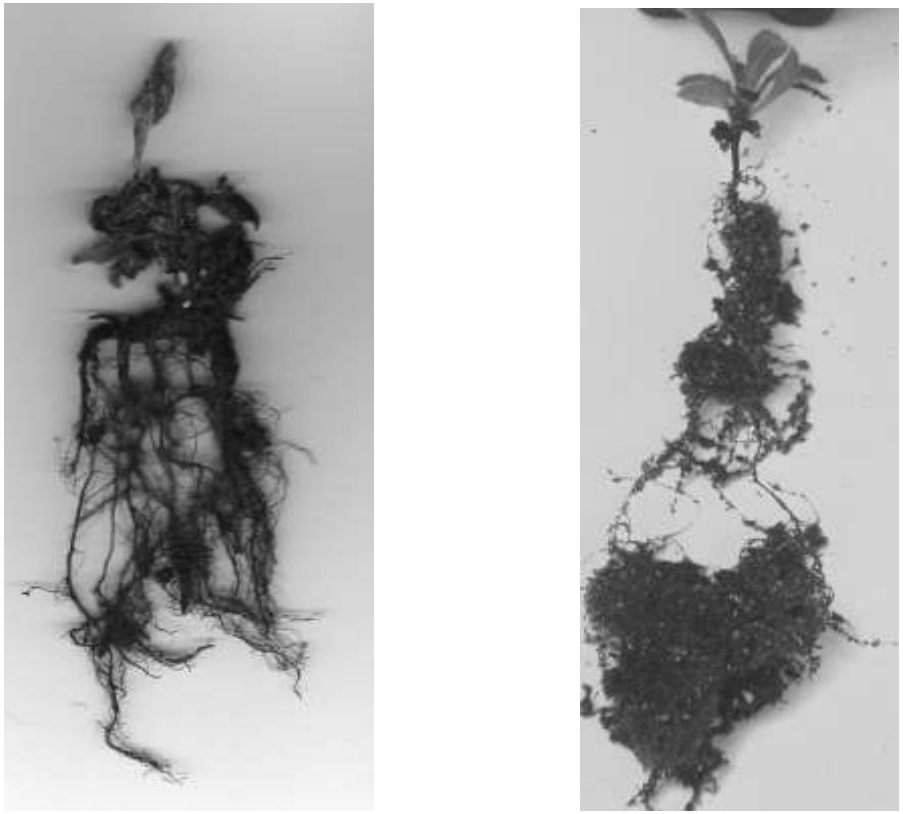

Figure A.1 *Hackelia venusta* roots. (L) divided taproot of older plant with further subdivisions (arrows) and fibrous roots. (R) with roots spread out from their original compact shape inside 2" pot. *J. Taylor*

Reintroduction and Outplanting Recommendations

If plants are being grown for reintroduction, it is probably a good idea to plant them out when they are small so they can recover from transplanting and establish a good root system. Since they need a constant source of moisture, it seems logical to pick either the late winter when the ground is still full of moisture, or if late season rains are timely, a fall planting could be tried if there is enough time for the plants to establish before freezing weather sets in. Supplemental watering is problematic because it would be difficult to enter the natural population often enough without damaging the habitat. A research population might be established near Forest Service offices so the plants could be tended, and protocols worked out for the best outplanting methods. In the habitat, it could be useful to try creating a more stable site by building an artificial rock garden, using large rocks that would allow repeated entry, or perhaps inserting small seedlings in existing rock crevices. Providing some shade when choosing a site is important, because it keeps plants cool and reduces stress. Four to six hours of sun each day is within the range of what plants receive naturally.

Plants should be located where their roots can grow into protected, moist areas - rocks provide an ideal mulch for this purpose. This is a common method to establish horticultural landscapes for other plants such as ferns that need evenly moist, cool soil. As discussed above, the original taproot tends to elongate as the plant ages, and spread along the soil surface or extend down into the soil. It may have leaves or a crown only on the growing tip. It appears that this thicker, tough root provides a strong connection between the leaves and the fine roots and may help the plant survive small slides in steep gravelly soils (Gentry and Carr 1976). Older plants that had been in larger pots had more than one division in the taproot. Divisions may not directly correlate with years of age, but they could be a way of aging plants in the field. Unfortunately, it would probably cause the death of the plant to excavate the taproot to the depth where divisions occur.

Another method for establishing plants is to plant or broadcast seeds directly. If seed is abundant, this is a more natural way to introduce plants in new areas, but it may not have a high success rate. If tried experimentally, it would be a good idea to plant seed in groups and make sure the spot could be relocated so that the germination rate and plant establishment could be monitored. Seeds should be planted or placed on the soil when they are naturally dispersing and checked before the end of winter for germination. Alternatively, they could be collected and then re-broadcast in the fall for exposure to moisture and cold. It may take several weeks for them to imbibe enough moisture. Unless they are frozen, they should begin to germinate about three months after thorough and constant wetting if they have enough cold weather.

Although members of the two *Hackelia* species I studied have adaptations such as stiff hairs on leaves for coping with arid conditions, and are almost pest- and disease-free in their natural environment, *H. venusta* seems to need certain protections to do well. Based on my observations in the field and from handling seedlings and plants in containers, I would suggest that seeds in the natural habitat probably germinate during mid-late winter when snow or rain provides a constantly moist seedbed. Roots can grow far enough in cold soil to reach moisture that will take them through the summer, and a few sets of leaves can develop by late March. In late winter, established plants have resprouted from dormant crowns, and usually start blooming by the third week in April. Since seeds disperse on the surface, it is unlikely they would be able to germinate after late winter or early spring and survive the heat of the summer. In the laboratory, a few seeds did germinate at cool spring and warm summer temperatures, so if local moisture is available, or enough rain falls, perhaps seedlings could germinate and grow during a wet period.

Average monthly low temperatures are lower in *H. venusta* habitat, and rainfall is higher than at lower elevation sites in the shrub-steppe environment further east. The steepness of the terrain ensures that at least part of the day many plants are in the shade because the sun is blocked by steep ridges. These factors promote lower

evaporation and transpiration and higher precipitation, thus a moister root zone over a longer period than that of the drier habitat to the east. The sun shines directly on the study site from a little after 10 am to about 4:30 pm during the longest days of summer. This could be important for maintaining soil moisture content and cooler leaf surface, air, and soil temperature. Mature plants in containers do best with evenly moist soil, which protects very fine fibrous roots that grow from thicker stems and roots near the crown. Root length is large in proportion to aboveground leaf area on seedlings grown in pots (Figure A.1).

It may be necessary to grow *H. venusta* in containers for reintroduction or research. Experience has shown that growing this species in containers successfully requires attention to their vulnerability to disease and pest damage, as well as still-ambiguous requirements for overwintering conditions. Seedlings can be grown successfully in cold to cool conditions, but keeping plants in pots alive outside a temperature-controlled environment has not been as successful. Those that do well in pots are much smaller than plants growing in natural conditions. Yearly fluctuations in weather could be a factor in plant establishment, reintroduction success, and size of seed crop. Late winter moisture and cool spring conditions should favor establishment and survival.

Weather Effects

In 2004, there was plentiful moisture early in the season before bloom, and surface runoff continued well into the blooming period. The weather was sunny and warm. In 2005 the winter was dry with little stored moisture or snow, and the spring was unusually dry and warm, followed by rain for about two weeks during peak bloom. Many companion plants such as *Dodecatheon spp.* seen in 2004 did not bloom at the same spot the second year, and mosses remained dry on boulders where seeps had kept them alive in April the year before. *H. venusta* plants were large and robust and seed set seemed heavy in 2004. My subjective impression was that really large plants were not as common in 2005. Pollinators are likewise affected by

weather. Therefore it seems reasonable to establish a monitoring program that tracks variable climatic conditions and population response, if any, over longer periods than just a few years. Permanent photo points, stem counts, seed counts, and pollinator data should be plotted with weather records to aid in long term monitoring of the population.

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PERSONAL COMMUNICATIONS

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