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ABSTRACT

PLANT EVOLUTION AND URBANIZATION: QUANTIFYING THE EFFECTS OF NATURAL SELECTION IN SHAPING SHEPHERD'S PURSE (*CAPSELLA BURSA-PASTORIS*) POPULATIONS IN NEW YORK CITY

by
Rebecca Panko

The aim of this study is to quantify the effects of natural selection in shaping *Capsella bursa-pastoris* populations along an urban-rural gradient in New York City.

A reciprocal transplant experiment with 168 lab-germinated *C. bursa-pastoris* seedlings from both urban and rural populations are grown in eight paired home and away sites distributed throughout the New York metropolitan area. Sites are visited approximately thirteen times to record plant fitness. There is evidence for local adaptation of urban populations: urban plants have longer reproductive durations and produce more seed pods in urban environments. These findings suggest that urban plants are better adapted to the stressful abiotic conditions found in urban areas.

Water stress laboratory trials test if urban populations are shaped by urban water stress regimes. The trials use 392 lab-germinated seedlings representing urban and rural populations from the New York metropolitan area, and include four water-stress treatments: drought, flood, cyclic drought and flood, and a well-watered control. Leaf traits from plants in the drought and control treatments are quantified to examine their role in water stress response. Both plant types appear unaffected by water stress, and demonstrate plasticity in leaf traits in response to drought. Leaf traits predict final plant size in the drought treatment but not in the control.

A salt stress trial tests if urban populations are shaped by urban soil salt stress. The trial includes 288 plants representing urban and rural populations from the New York metropolitan area. Plants are grown under different salt treatments (0, 20, 40, 50, 60, 100, and 150 mM NaCl) for five weeks. Both plant types demonstrate salt-sensitivity, having high rates of mortality at high salt concentrations. However, plants that survive high salt treatments are significantly larger than controls, indicating some individuals are salt tolerant. Leaf trait analysis demonstrates that different plastic responses occur in plants grown in salt stress compared to those grown in drought.

The reciprocal transplant experiment shows evidence of local adaptation in urban populations, whereas the laboratory trials find that the species is highly plastic in leaf trait responses to drought and salinity.

PLANT EVOLUTION AND URBANIZATION: QUANTIFYING THE EFFECTS OF NATURAL SELECTION IN SHAPING SHEPHERD'S PURSE (*CAPSELLA BURSA-PASTORIS*) POPULATIONS IN NEW YORK CITY

by
Rebecca Panko

A Dissertation
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APPROVAL PAGE

PLANT EVOLUTION AND URBANIZATION: QUANTIFYING THE EFFECTS OF NATURAL SELECTION IN SHAPING SHEPHERD'S PURSE (*CAPSELLA BURSA-PASTORIS*) POPULATIONS IN NEW YORK CITY

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This work is dedicated to my grandmother, Mary Rose Panko.

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TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

TABLE OF CONTENTS
(Continued)

TABLE OF CONTENTS
(Continued)

TABLE OF CONTENTS
(Continued)

Chapter **Page**

LIST OF TABLES

LIST OF TABLES
(Continued)

LIST OF FIGURES

LIST OF FIGURES
(Continued)

LIST OF FIGURES
(Continued)

LIST OF FIGURES
(Continued)

CHAPTER 1

INTRODUCTION

1.1 Background

Urban areas are expanding rapidly. Human activity plays a central role in climatological and environmental changes (Steffen, Crutzen, & McNeill, 2007). Urban areas are associated with a syndrome of environmental changes including increased habitat fragmentation, habitat degradation, and altered abiotic/biotic factors (Johnson, Thompson, & Saini, 2015). These changes have significant negative impacts across taxa (Aronson et al., 2014).

Plant species in urban areas contend with altered abiotic factors including highly variable soil moisture and increased soil salinity (Gaston, 2010). Increased impervious surface prevents soil infiltration and increases flash flooding events (Boyle, Lavkulich, & Schreier, 1997; McPhearson, Hamstead, & Kremer, 2014), exposing urban plants to episodic drought and inundation. Application of de-icing salt increases soil salinity near roadsides and in street-tree pits (Cekstere & Osvalde, 2013). These urban abiotic factors can cause urban plant populations to be selected to tolerate higher salinity values and/or altered hydrological regimes.

There is mounting evidence that urbanization is driving evolutionary processes in various taxa. Urban populations of white-footed mice (*Peromyscus leucopus*) in New York City carry genes for heavy metal tolerance and increased immune response (Munshi-South, Zolnik, & Harris, 2016). Urban populations of nocturnal orb-web spiders (Araneidae) show preference for nest sites near artificial light sources (even in lab-reared individuals with no

previous exposure to artificial light) (Heiling, 1999). Populations of lizards (*Anolis cristatellus*) have undergone phenotypic shifts that make urban lizards better able to climb artificial surfaces (Winchell, Reynolds, Prado-Irwin, Puente-Rolan, & Revell, 2016).

For urban plants, we see evidence that urbanization is driving evolutionary processes, particularly in weedy, early-successional species. Cheptou and colleagues (2008) demonstrated that *Crepis sancta* – a species that bears both tufted seeds that are wind-dispersed and non-tufted seed morphs on the same plant – produces significantly more non-tufted seeds in fragmented urban populations compared to less-fragmented rural populations. Annual meadow grass (*Poa annua*) has adapted to mowing regimes by exhibiting a shorter stature at maturity (Velguth & White, 1998). More recently, Thompson and colleagues (2016) found that urban populations of white clover (*Trifolium repens*) have reduced cyanogenesis relative to non-urban populations in three cities (New York, NY, Boston, MA, and Toronto, Canada). Cyanogenesis is a Mendelian-inherited trait that helps protect plants from herbivory (cyanogenic plants produce hydrogen cyanide following tissue damage).

Urbanization alters the abiotic environment, potentially driving plant evolution via natural selection. Natural selection is a non-random process, and the survival and reproductive success of individuals are directly influenced by an organism's environment. For a simplified example, imagine a hypothetical population of plants that resides in a low-salt environment. If there is an influx of salt into the environment (Figure 1.1, A), perhaps due to seasonal de-icing salt application, plants with the lowest tolerance to salt would not be as fit as plants with higher tolerance. After ten generations, the number of salt-tolerant individuals might increase further (Figure 1.1, B). If the soil salinity continues to remain

elevated over generations, selection pressure could result in a population entirely composed of salt-tolerant individuals (Figure. 1.1, C).

If natural selection is occurring along the urban-rural gradient, plant populations will be locally adapted. A reciprocal transplant experiment directly tests for local adaptation by comparing the relative fitness of individuals when grown at their home site and a paired away site (Savolainen, Pyhäjärvi, & Knürr, 2007; Franks, Weber, & Aitken, 2014). Laboratory trials can also be used to compare the relative fitness of individuals grown under different environmental stress treatments. Relative fitness can be estimated by comparing differential reproductive success (e.g., number of flowers, number of seeds produced). In the absence of reproductive structures, biomass (e.g., final plant size) or growth rate can be used as a proxy for fitness (Younginger, Sirova, Cruzan, & Ballhorn, 2017).

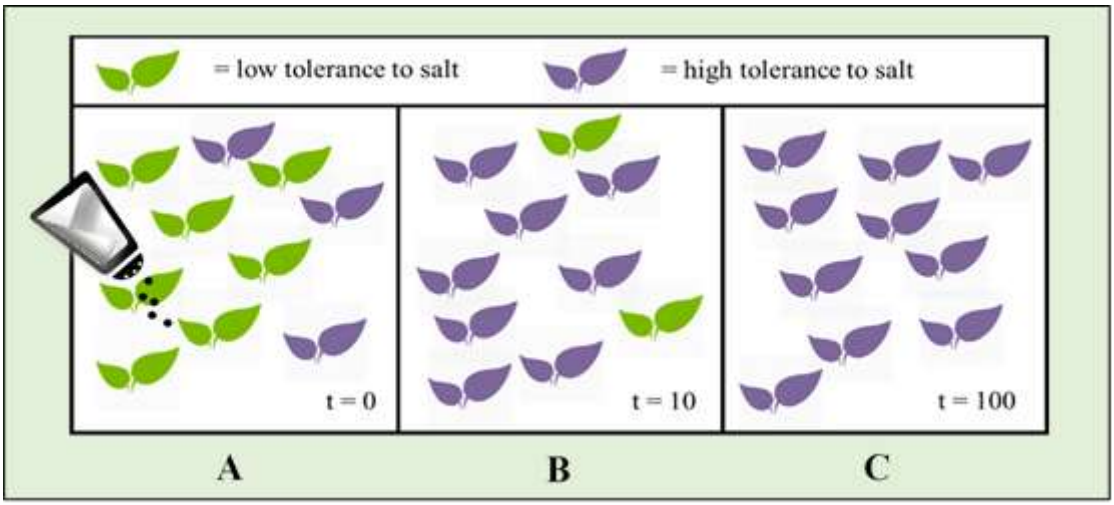

Figure 1.1 A simplified example of natural selection shaping plant populations in urban environments. (A) A hypothetical plant population that resides in soil with low salt concentrations experiences an input of salt. (B) Over generations (t =10) the proportion of salt-tolerant individuals in the population increases. (C) If salt inputs continue over additional generations (t = 100), the population may be entirely comprised of salt-tolerant phenotypes. In this example, natural selection is the primary driver shaping this urban plant population.

There have been several studies on our theoretical understanding of urbanization's ecological implications (Aronson, Handel, La Puma, & Clemants, 2015; Aronson, et al., 2016; Hobbs, Higgs, & Harris, 2009; Johnson & Munshi-South, 2017; Johnson et al., 2015; McDonnell & Hahs, 2015; McDonnell & Pickett, 1990). There have been great efforts towards understanding how wild plant populations within and around cites evolve (Cheptou, Carrue, Rouifed, & Cantarel, 2008; Cheptou, Hargreaves, Bonte, & Jacquemyn, 2017; Donihue & Lambert, 2015; Dubois & Cheptou, 2017; Gorton, Moeller, & Tiffin, 2018; Grimm et al., 2008; Lambrecht, Mahieu, & Cheptou, 2016; Thompson, Renaudin, & Johnson, 2016; Yakub & Tiffin, 2017). However, as is often the case in ecology and evolution, our theoretical understanding currently exceeds actual evidence gained from hypothesis testing. Thus, there is a great need for field and laboratory studies to test for the effects of different evolutionary mechanisms in urban environments.

1.2 Objective

The goal of this work is to quantify the relative fitness of urban and rural populations of shepherd's purse (*Capsella bursa-pastoris*) in the New York metropolitan area to determine if populations are locally adapting to conditions along the urban-rural gradient. From an evolutionary standpoint, this work addresses these key questions:

1. Are wild plant populations locally adapting to the increased abiotic stress found in urban areas?
2. If they are locally adapted, which environmental factors are primarily responsible?

1.3 Main Hypothesis

We hypothesize that urban populations have undergone adaptive evolution in abiotic stress response via prolonged exposure to urban abiotic stresses (e.g., episodic drought/inundation and increased soil salinity). We test this hypothesis in a series of experiments, a reciprocal transplant experiment in the New York metropolitan area and laboratory abiotic stress trials.

1.4 Study Species

Capsella bursa-pastoris (L.) Medik. (Brassicaceae) (shepherd's purse) (Figure 1.2) is a small herb, roughly 0.7 m in height. The name refers to seed pods that resemble small purses. A cold-season annual, the species flowers rapidly, self-pollinates, and produces copious amounts of seed. It is a relatively young species (~100-300 thousand years old), originating from Eastern Europe and Western Asia (Douglas et al., 2015). It is a very successful weedy species, having a nearly worldwide distribution (Figure 1.3). An early-colonizing species, it is commonly found in disturbed sites most likely to experience increased abiotic stress like higher soil salinity and highly variable soil moisture (Douglas et al., 2015). It often grows in locations such as sidewalk cracks, along roads, in street tree pits, and in gardens. It is short-statured and has short generation times, making it a good choice for both reciprocal transplant experiments and laboratory trials (Neuffer & Eschner, 1995).

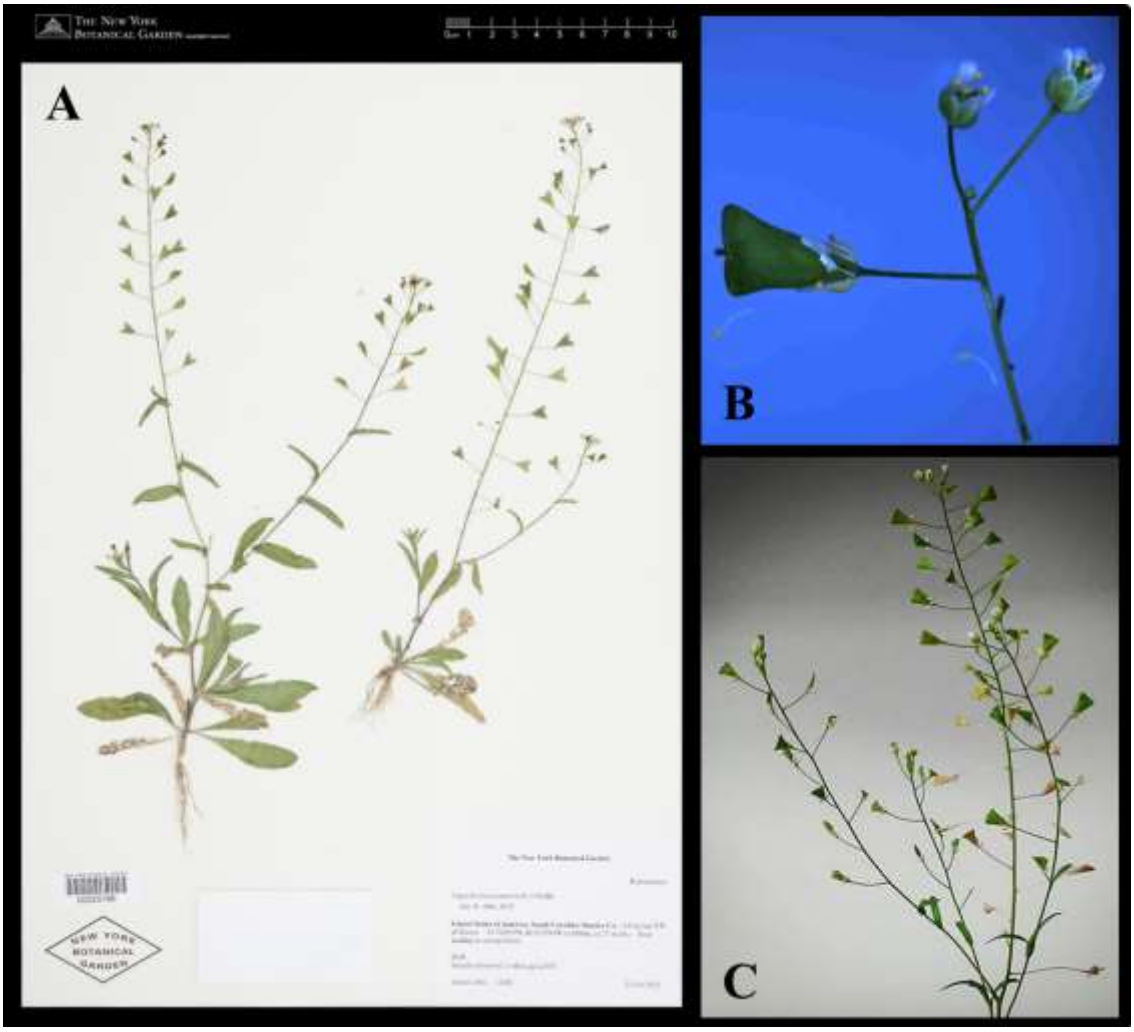

Figure 1.2 Overview of study species, *Capsella bursa-pastoris* (shepherd's purse): (A) herbarium specimen of two plants with basal rosette and infructescences (Atha, 2012), (B) detail of flowers and seed pod, and (C) detail of living infructescence. Photos by Rebecca Panko.

1.5 Significance

There are several on-going research efforts across the discipline that aim to identify how plant species adapt to urban conditions. To date, the literature has included the following methods: greenhouse experiments, common garden experiments, wild plant accessions, and genetic analysis. In the field of urban evolutionary biology, one noticeably absent methodology is the use of reciprocal transplant experiments, despite its frequency in other

ecological disciplines (Franks et al., 2014). To our knowledge, only one published study (Gorton et al., 2018) used reciprocal transplant experiments to measure adaptation in response to urbanization. Therefore, our experimental design is relatively rare in the burgeoning field of urban evolutionary biology.

1.6 Relevance to Other Disciplines

In addition to being relevant to urban ecologists and evolutionary biologists, our work is accessible across a wide range of disciplines. We describe environmental consequences of urbanization, and quantify the magnitude of urbanization of sites using GIS and publicly accessible land cover data. We provide soil heavy metal concentration data for urban and rural sites, and describe other environmental conditions of these sites (e.g., vegetation types, soil compaction, and canopy cover). These techniques have applications for fields including environmental consultation, city planning, and other ecological, physiological, and soil-science disciplines.

Other aspects of our work are relevant to fields such as botany, plant-science, and other plant-related disciplines. We propose plant strategies that might best adapt to urban conditions, perform field surveys, reciprocal transplant experiments, common-garden laboratory experiments, and present plant fitness data. We also discuss the botanical characteristics of our species, and connect life-history traits and morphology to increased adaptive potential. Our statistical analyses include using mixed-models and model fitting, techniques applicable to a wide-range of interdisciplinary fields. Lastly, we use a variety of instruments to perform our work, including tools commonly used in forestry and

agriculture (e.g., soil penetrometer, soil moisture sensors) and even common household items (e.g., toothpicks and plastic forks).

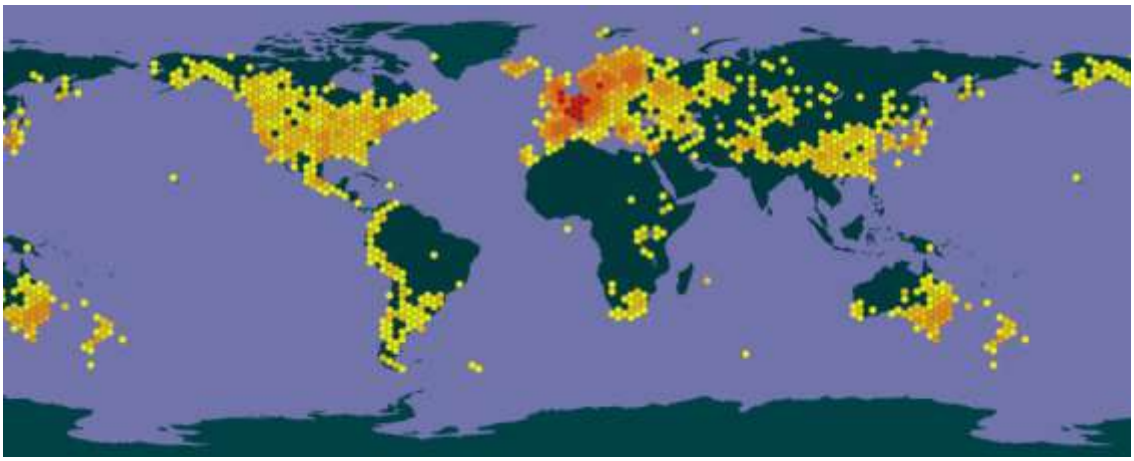

Figure 1.3 Worldwide distribution occurrence heat-map of *C. bursa-pastoris* (shepherd's purse) (from years 1600-2020) on GBIF https://www.gbif.org/species/5375388 (accessed on 4.14.2020).

CHAPTER 2

EVIDENCE OF LOCAL ADAPTATION IN *CAPSELLA BURSA-PASTORIS* ALONG AN URBAN RURAL GRADIENT IN NEW YORK CITY

2.1 Overview

Life in the city is stressful, yet some plant species have succeeded spectacularly in these human-dominated landscapes, achieving global distributions. Cities experience changes in abiotic factors that may be driving the evolution of wild urban plant populations. Here, we quantify the effects of natural selection in shaping *Capsella bursa-pastoris* along an urban-rural gradient in New York City.

We hypothesize that urban populations have undergone adaptive evolution in response to abiotic stress in urban environments, and predict that plants from urban populations will demonstrate higher relative fitness at urban sites compared to rural sites. To test this hypothesis we conducted a reciprocal transplant experiment, transplanting 168 lab-germinated *C. bursa-pastoris* seedlings from both urban and rural populations into eight paired home and away sites distributed throughout the New York metropolitan area. The paired sites were selected from 24 candidate sites based on germination trials, site safety and accessibility, and categorized into 'urban' and 'rural' based on proportion impervious surface. We revisited each site approximately 13 times to record plant fitness.

We found that 1) plants lived shorter lives at urban sites, 2) urban plant flowering onset was earlier at urban sites, 3) plants had significantly longer reproductive duration at their home sites, and 4) urban plants produced more seed pods at urban sites. Our findings suggest that urban plants are better adapted to the stressful abiotic conditions found at urban sites, and support our hypothesis that adaptive evolution is shaping urban and rural

populations of *C. bursa-pastoris* along this urban-rural gradient. Cities present a multitude of abiotic stress factors to wild plant populations, and it is unclear which factor plays the largest selective role for this cosmopolitan weed.

2.2 Background

Increasing urbanization is a reality of the Anthropocene. The percentage of humans that live in cities has nearly doubled over the last century, rising from 30% in 1950 to 55.3% in 2018 (United Nations, 2014 & 2018). Indeed, the far-reaching effect of human activities at different spatial scales is present across global ecosystems (Steffen et al., 2007; Vitousek, Mooney, Lubchenco, & Melillo, 1997). The ecological and evolutionary consequences of human-dominated landscapes are still largely unknown, and efforts to resolve these questions have increased dramatically over the last several decades (Donihue & Lambert, 2015; Grimm et al., 2008; Johnson & Munshi-South, 2017). In particular, much work has focused on how urbanization is driving the evolution of wild plant species (Cheptou et al., 2008; Cheptou et al., 2017; Dubois & Cheptou, 2017; Gorton et al., 2018; Lambrecht et al., 2016; Thompson et al., 2016; Yakub & Tiffin, 2017).

Cities are characterized by high human population density and built infrastructure (Alberti, 2015), which create novel ecosystems via ecological and environmental changes (Hobbs et al., 2009). Urban areas have high rates of habitat destruction, fragmentation, and altered disturbance regimes (Aronson et al., 2015; McDonnell & Pickett, 1990). Cities have increased proportion of impervious surfaces, higher relative air temperature, and more pollution compared to rural locales (Johnson & Munshi-South, 2017). Plant populations in urban areas must also contend with increased nutrient and water stress, increased soil

compaction, and soils with high salinity and heavy metal concentrations (Del Tredici, 2007; Grimm et al., 2008).

One key question is how urbanization impacts selective evolutionary processes that shape urban plant populations. The abiotic stress in urban environments might be so intense that persistent populations adapt via selection over very few generations. Indeed, rapid evolution has been documented in fragmented urban populations of the weedy species *Crepis sancta* (Cheptou et al., 2008). There are several on-going research efforts to identify how plant species adapt to urban conditions. A recent review by Johnson and Munshi-South (2017) highlights evidence that adaptive evolution has led to divergent selection of urban and non-urban plant populations, including differences in reproductive traits (Cheptou et al., 2008; Dubois & Cheptou, 2017; Yakub & Tiffin, 2017) and physiology (Lambrecht et al., 2016; Thompson et al., 2016). These studies employed strategies including common garden/greenhouse experiments, wild plant accessions, and genetic analyses. To date, reciprocal transplant experiments are largely absent from studies of plant adaptation along urban-rural gradients, despite its acceptance as a robust test for local adaptation and its frequency in other ecological disciplines (Franks et al., 2014). Reciprocal transplant experiments test for local adaptation as shown by higher relative fitness (e.g., seed production) at their home sites compared to away sites (Savolainen et al., 2007; Franks et al., 2014). To our knowledge, only one published study (Gorton et al., 2018) used reciprocal transplant experiments to explicitly measure adaptation in response to urbanization. They found evidence that urban and rural populations of *Ambrosia artemisiifolia* are diverging in flowering time, but also implicate the role of genetic drift. Indeed, the highly fragmented nature of cities isolates populations, which could result in

smaller populations with limited gene flow (Savolainen et al., 2007). There is still much we do not understand about the interplay of these evolutionary mechanisms in urban environments, but here we focus on the role of selection.

Specific life-history traits and plant strategies may influence the likelihood that plant species adapt to stressful urban conditions. Species that have short generation times and good seed dispersal ability (McDonnell & Hahs, 2015), as well as high tolerance to pollution, soil compaction, and trampling (McKinney, 2006) are good candidates for long-term residence in cities. In particular, ruderal species prone to disturbed habitats that also display short habits (e.g., rosette or semi-rosette form) have increased occurrence in urban environments (Vallet, Daniel, Beaujouan, Rozé, & Pavoine, 2010; Williams, Hahs, & Vesk, 2015). In terms of plant niche extremes (Grime, 1977), populations of plant species with these attributes may better withstand life in urban niches that are characterized by high abiotic stress (e.g., altered temperatures, hydrological regimes, and polluted soils) and high rates of disturbance (e.g., trampling, mowing).

Capsella bursa-pastoris (L.) Medik. (Brassicaceae) (shepherd's purse) is a cosmopolitan weed with many of the characteristics described above. Named for its purse-shaped seed pods (Figure 2.1), it is a small (height 0.7 m), cold-season annual with rosette habit, often found in disturbed habitats like roadsides, sidewalk cracks, and crop fields. The species predominantly self-pollinates and is a prolific seeder, with reports of 11,000-400,000 seeds produced per square-meter (Hill, Renner, & Sprague, 2014). The species originated in Eurasia, and its recent worldwide distribution correlates with European colonization (Hurka & Neuffer, 1997). A mucilaginous seed coat may improve dispersal

ability (Neuffer, Bernhardt, Hurka, & Kropf, 2011), particularly in paved urban settings with high foot-traffic.

Figure 2.1 Example urban habitats of *Capsella bursa-pastoris*, showing (A) detail of flower inflorescence and (B) growth habit. Photos by Rebecca Panko.

We propose that these life history traits and its predominance in cities make *C. bursa-pastoris* a good candidate for examining whether plant populations are locally adapted to urban conditions. Indeed, the species has been shown to demonstrate early and late flowering genotypes across latitudinal gradients (Neuffer et al., 2011). Phenotypic variability is common in the species, with several leaf, flower, and seed pod variants occurring naturally (Eldridge et al., 2016; Neuffer, Wesse, Voss, & Scheibe, 2018; Ziermann et al., 2009). This variability may be due to duplicate gene expression (Adams, 2007; Neuffer & Eschner, 1995); the species is an allotetraploid thought to have resulted from a hybridization event between other members of *Capsella* (Cornille et al., 2016).

To quantify the ability of plants to adapt to urbanization, we conducted a reciprocal transplant experiment along an urban-rural gradient in New York City using populations of *C. bursa-pastoris*. We hypothesize that urban populations have undergone adaptive

evolution in abiotic stress tolerance via prolonged exposure to abiotic stress in urban environments, and predict that plants from urban populations will demonstrate higher relative fitness in urban environments than in rural environments.

2.3 Materials and Methods

2.3.1 Study Site and Seed Collection

We conducted the study along an urban-rural gradient in the New York metropolitan area, defined as a 60-km radius from Times Square (40°75'N, 73°98'W). Seed collection surveys occurred May-June 2017. We chose intermediate points along the urban-rural gradient using a stratified random approach. Surveyors performed surveys for roadside populations of *Capsella bursa-pastoris* by following local roads that radiated out from each randomly established point until a population was located.

The following data were collected at each population: habitat type, population area dimensions, proportion of area sampled for seed pods, number of plants in population, percentage of plants producing seed pods, dominant vegetation cover, and whether the site was a good candidate for subsequent reciprocal transplant trials. Seed pods were harvested from visibly healthy plants (n = 15; hereafter, "parent plants") at each population (n = 24) (Figure 2.2). Seed pod infructescences were cut from the base of each plant using scissors and placed into paper envelopes. The envelopes were labeled with parent plant voucher number, GPS coordinates, and population number. The envelopes were transported to New Jersey Institute of Technology and placed in a dry, well-ventilated location.

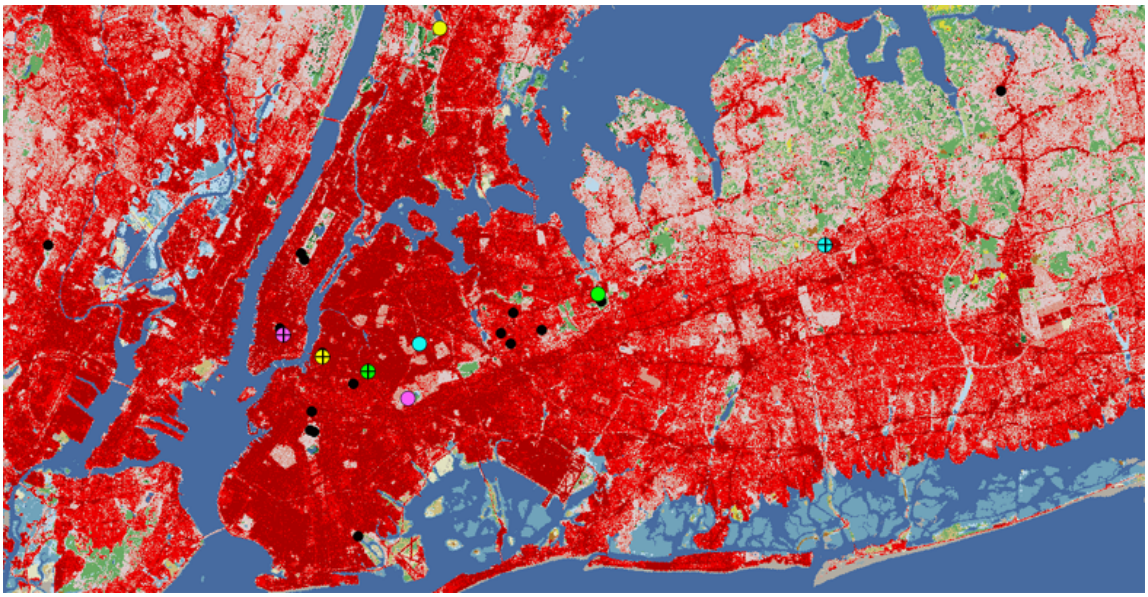

Figure 2.2 Map of the study site showing locations of reciprocal transplant paired sites (colored dots indicate pairs) and additional sites where seed collections occurred (black dots). Impervious surface (IS) land cover (30 m resolution) are from the National Land Cover Database 2011. We calculated IS at a local scale (0.0081 km^2) for each reciprocal transplant site: urban sites = IS_{local} > 70% (crossed dots), rural sites = IS_{local} < 50% (uncrossed dots) (green dots) Site 1 (72.5 IS_{local}) & Site 2 (24.2 IS_{local}), (yellow dots) Site 3 (33.6 IS_{local}) & Site 4 (87.2 IS_{local}), (pink dots) Site 5 (87.2 IS_{local}) & Site 6 (9.9 IS_{local}), (blue dots) Site 7 (81.5 IS_{local}) & Site 8 (41.2 IS_{local}).

We analyzed the local proportion impervious surface (0.0081 km^2) surrounding each population using QGIS (Version 2.18.3) (QGIS Development Team 2017), and 30 m resolution impervious surface land cover data from the 2011 National Land Cover Database (Multi-Resolution Land Characteristics Consortium www.mrlc.gov/data accessed on 21 February 2017). We imported our population coordinates, and quantified local proportion impervious surface as the average proportion impervious surface of a population's pixel and the surrounding 8 pixels (Figure 2.2). We used the local impervious surface to designate reciprocal transplant pairs: urban sites = IS_{local} > 70%; rural sites = IS_{local} < 50%.

2.3.2 Germination Trials and Reciprocal Transplant Experiment Preparation

We ran a germination trial using seeds from population sites that were good candidates for reciprocal transplant experiments in a climate controlled vegetation room at the New Jersey Institute of Technology. Candidate sites were chosen based on proportion impervious surface and site safety and accessibility (Figure 2.2). The germination trial (January 2018) included seeds (n = 988) representing 47 random parent plants from 11 candidate populations (urban = 7, rural = 4). Seeds were sown into 4-pack seed-starting containers (806 Inserts, Grower's Solution, Cookeville, TN, USA) in standard (55 cm x 28 cm) plant trays on metal plant shelves (Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA).

We used a fast-draining, high porosity soil (Pro-Mix HP, Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA) and a 12 h photoperiod during the trial. Germinated seedlings were kept under germination hoods beneath grow lights (Sun Blaze 44, ACF Greenhouses, Buffalo Junction, VA, USA) and plant trays were rotated twice a week. Visible seedlings were counted after two weeks. Additional seeds (n = 7160) representing 75 random parent plants from 13 candidate populations (urban = 5, rural = 8) were sown (February 2018) as stock for the reciprocal transplant experiments. To improve germination rates, we used a germination mix (LM-18 Germination Mix, Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA) and a 16 h photoperiod. We monitored humidity and temperature of the vegetation room to ensure an adequate growth environment (Humidity and Temperature Smart Home Environment System, AcuRite, Lake Geneva, WI, USA).

Reciprocal transplant seedlings were grown in the vegetation room under germination hoods and rotated twice a week for three weeks. Seedlings from each pair of

sites were inventoried and vouchered (e.g., population ID, parent plant ID, seedling ID). The three-week old seedlings were transported to a backyard in Brooklyn, New York and allowed to acclimate under germination hoods until weather conditions permitted planting at the reciprocal transplant sites (time in Brooklyn ~17 days). When planting the seedlings into the field sites, we selected seedlings such that each parent plant could be represented by at least two seedlings at each site in a pair of sites, whenever possible.

2.3.3 Reciprocal Transplant Trials

The reciprocal transplant paired sites were planted in March 2018 as follows: Site 1 and 2 on March 26, site 3 and 4 on March 28, site 5 and 6 on March 30, and site 7 and 8 on March 31 (Figure 2.2). An example site pair is shown in Figure 2.3. At each site, seedlings were drawn from both the home and away populations and from each parent plant within those populations in a stratified random manner and assigned to planting positions within a four by five grid at each site (2 populations * 5 parent plants * 2 seedlings per parent plant). Each location in the grid was assigned a unique color code, with the same code used at each site. We used color-coded wooden toothpicks to identify plants in the field during the experiment.

Upon arriving at a transplantation site, we established a temporary 125 cm x 150 cm plot marked with flagging. We recorded weather data including sunny/cloudy, precipitation, wind, and ground temperature in the shade. We estimated the tree canopy cover over the plot and the percent ground cover of herbaceous vegetation within the plot, organic litter, garbage litter, and bare soil in the plot. We removed any large bulky garbage from the plot. We recorded whether the plot was in danger of foot traffic, whether garbage or pet excrement was visible, and if any *C. bursa-pastoris* was present. To characterize soil

conditions, we collected three measurements of each of the following: compaction (soil penetrometer, Lang Penetrometer, Inc., Gulf Shores, AL, USA), temperature (Polder Stable Read Digital Thermometer, Polder Products, LLC, Oxford, CT, USA), and moisture (direct, mineral, and organic) (ThetaProbe ML2x, Delta-T Devices, Cambridge, England). We collected soil from the top 10 cm of the surface from five locations (e.g., corners and center) in the plot to produce a representative 4-cup sample for soil heavy-metal analysis (performed by the Rodríguez-Freire lab at NJIT) (see Appendix A.1, Analytical Methods).

We estimated site topography features including distance to and type of impervious surface (e.g., road, sidewalk), whether the impervious surface was upslope or downslope from the plot, and whether water could drain from the impervious surface onto the site.

Figure 2.3 Example of a reciprocal transplant site pair. (A) Site 3 in Yonkers (rural) and (B) Site 4 in Williamsburg, Brooklyn (urban). Photos by Rebecca Panko.

We recorded the following when planting each seedling: date, seedling voucher, number of leaves, chlorotic leaves, damaged leaves, longest leaf, the lengths of the primary and secondary axes of the basal rosette, and color-coded location in the planting grid. Each seedling was carefully removed from its plastic pot, loose dirt gently removed from roots,

and roots dipped into a small water bath to remove as much propagation soil as possible without damaging the plant. We used a small plastic fork to make a hole in the plot and placed the seedling in the center, gently filling in soil until the seedling was secure. We watered the seedling (10 ml) and labeled it with the color-coded toothpicks. Seedlings were planted 25 cm apart in the plot in the order of the stratified random seedling voucher numbers described above. Our initial experimental design called for twenty seedlings per site, representing 10 parent plants (e.g., five from each site of paired sites). However, despite best efforts, fewer seedlings were planted at some sites due to lack of plant material. The number of seedlings planted at each site on planting day was: sites 1–4 and site 6 (n = 20, per site), site 5 (n = 19), site 7 (n = 18), and site 8 (n = 17). We placed remote soil temperature sensors (iButton, Embedded Data Systems, Lawrenceburg, KY, USA) at each site buried 10 cm deep within small vacuum-sealed bags (FoodSaver, Oklahoma City, OK, USA) 25 cm from the plot, triangulating its location for later retrieval. We sketched and photographed the finished plot (including permanent fixtures and temperature sensor) to facilitate finding the seedlings on site revisits.

We revisited each site to record fitness data on the following days after planting: 3, 6, 8, 10, 14, 21, 28, 35, 42, 49, 60, 70, and 80. Revisits during the first four weeks recorded whether plants were alive or dead and longest leaf. Revisits after that time recorded the following: alive or dead, longest leaf, number of leaves, number of flowers, number of seed pods, number of primary and secondary flower stalks, number of open seed pods, number of stunted seed pods, and plant width and height. Truck tire damage at site four occurred between planting day and day three; fourteen replacement plants were planted at that site (four on day six, ten on day eleven). Hemispherical photos were taken after canopy plants

leafed out to characterize the light environment (Nikon D90 camera, Minato City, Tokyo, Japan; Sigma Corporation of America Circular Fisheye DC HSM 4.5mm 1:2.8, Ronkonkoma, New York, USA). From the photos we calculated canopy cover over each plot (ImageJ 1.47) (Schneider, Rasband, & Eliceiri, 2012). Steps for processing images were as follows: 1) upload image, 2) isolate lens area using the ellipse tool, 3) crop the image, 4) make the image binary, 5) analyze the results using histogram, 6) download binary pixel counts (Beckschäfer, 2015). Temperature sensors (when recovered) were collected on day 50 and returned to the lab for analysis.

2.3.4 Statistical Analyses

Because our experimental design included random effects for both parent plant and site pair, we employed a mixed model analysis using R package lme4 (Bates, Mächler, Bolker, & Walker, 2014) in R (R software v.3.5.1, R Core Team, 2019).We first fitted a full model including plant type (urban or rural), planting location (home or away), and their interaction as fixed effects, and parent plant and site pair as random effects. We reduced these full models by removing the random effects in turn, using AIC (Akaike, 1974) to select the best fitting model.

We used R package lmerTest (Kuznetsova, Brockhoff, & Christensen, 2017) to conduct F-tests for fixed effects using the Satterthwaite approximation for denominator degrees of freedom (Satterthwaite, 1946).

To explicitly compare responses of each plant type to home and away planting sites, we conducted orthogonal contrasts in R package emmeans (Lenth, 2019) using Satterthwaite denominator degrees of freedom. Finally, we used R package emmeans to extract estimated marginal means (Figures 4-7). When presenting our results we focus on

effect sizes and confidence intervals rather than p-values and statistical significance. This approach follows current best practices in both ecology and evolution (Dushoff, Kain, & Bolker, 2019) and statistics (Wasserstein, Schirm, & Lazar, 2019), and allows readers to interpret effect sizes and p-values for themselves. Response variables were log-transformed when necessary to meet the assumptions of normality and homoscedasticity, and estimated means were back-transformed for presentation in figures.

2.4 Results

2.4.1 Study Site and Seed Collection

The broader pool of 24 populations sampled for seeds occurred along roadsides (58%) and in public parks (42%). In addition to *C. bursa-pastoris*, dominant plants along roadsides were mostly ruderals (e.g., *Lepidium virginicum*, *Plantango* sp., *Poa* sp.), planted street trees (e.g., *Zelkova* sp., *Tilia* sp.), and wild woody plants (e.g., *Ailanthus altissima*, *Morus* sp.). Dominant plants in parks included ruderal species mentioned above and others (e.g., *Taraxacum* sp.), many ornamentals (e.g., Rosaceae, *Cedrus* sp.), common northeastern trees (e.g., *Quercus* sp., Pineaceae), and woody vines (e.g., *Toxicodendron radicans*). The local proportion impervious surface (0.0081 km^2) of roadside populations was much higher than park populations (+25.4% ±7.2%). The mean population area was 82 m^2 (range 1–930 m^2), and mean proportion of area sampled for seeds was 17 m^2 (range 1–220 m^2). Mean population size was 90 plants, with populations having as few as fifteen or over a thousand individuals. All populations had 100% of plants producing seed when seed sampling occurred.

2.4.2 Germination Trials and Reciprocal Transplant Experiment Preparation

The percent germination rate was low for both trials (January: 4.5% ± 1.4%; February: 7.8% ± 1.2%), whether seeds were collected from rural sites (January: 5.6% ± 1.7%; February: 10.6% ± 1.8%) or urban sites (January: 3.7% ± 2%; February: 3.4% ± 0.8%).

2.4.3 Reciprocal Transplant Trials

Because each site pair was planted on the same day, weather conditions at the time of planting were similar within each pair, except for paired sites five and six: conditions at site five were partly sunny whereas it was raining at site six. Air temperature in the shade was higher at urban sites on average (+2.7°C ± 1.95). Pet excrement and/or garbage was present in 50% of rural sites and 100% of urban sites. Most urban sites (75%) were in danger of foot traffic, compared to only 50% of rural sites. *Capsella bursa-pastoris* rosettes were present at 25% of both urban and rural sites at the time of planting, but not present within any of the experimental plots. Urban sites had more organic litter (+15.5% ± 18.3%) and bare soil (+17.3 ± 2%), whereas rural sites had more greenery (+32.7% ± 14.2%) (See Figure A.2) and denser canopy cover (+8.8% ± 10.6%).

Soil temperature (°C) and direct probe moisture (V) were similar at urban (9.275°C ± 0.97; 0.7025 V ± 0.064) and rural sites (8.392 °C ± 0.41; 0.78 V ± 0.065), however soil compaction was higher at urban sites (+1.61 kg ± 0.59) (Figure A.3). Soil heavy metal analyses (Table A.4) showed that average metal concentration was very similar at both urban and rural site types. Many of the soil temperature sensors were lost and not recovered, and we were only able to obtain comparison data for one site pair (rural site 3

and urban site 4); soil temperature was similar at both sites for the observed period (March 28th – May 17th).

2.4.4 Effects on Fitness Components

Both urban and rural plants survived roughly twice as long in rural sites than in urban sites (Urban: +35 days ± 5.0 se, t-ratio = -7.06, p < .0001; Rural: +28 days ± 4.7 se, t-ratio = 6.00, p <.0001; Figure 2.4; Tables 2.1 and 2.2). Urban plants took longer to produce flowers when planted in away sites (+19.7 days ± 4.1 se, t-ratio = 4.76, p < .0001) while rural plants did not differ substantially in days to first flower at away sites compared to home sites (+3.0 days ± 3.5 se, t-ratio = -0.87, p = .3859; Figure 2.5; Tables 2.1 and 2.2). Urban plants flowered roughly twice as long at home sites than at away sites (+11.1 days ± 5.0, t-ratio = 2.23, p = .0291) while rural plants flowered roughly 50% longer at home (+6.8 days ± 4.1 t-ratio = 1.64, p = .1058) (Figure 2.6, Tables 2.1 and 2.2). Urban plants produced nearly four times as many seed pods at home sites compared to away sites (t-ratio = 2.04, p = .0448), whereas rural plants produced only 20% more seed pods at home sites than at away sites (t-ratio = -0.36, p = .7186) (Figure 2.7). However, Type III F-tests for the full model suggest marginal clarity for effects on seed pod production, suggesting further experimentation is needed to fully characterize this effect (Tables 2.1 and 2.2).

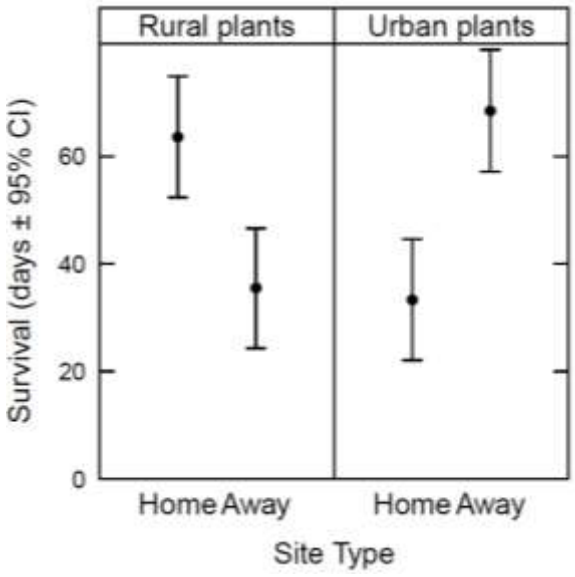

Figure 2.4 Effect of plant type (rural vs. urban seed origin) and planting location (home site vs. away site) on survival duration. Both urban (35 days ± 5.0 se, t-ratio = -7.06, $p < .0001$) and rural (28 days ± 4.7 se, t-ratio = 6.00, $p<.0001$) plants survived roughly twice as long in rural sites than in urban sites (see Table 2.1 for Type III F-tests).

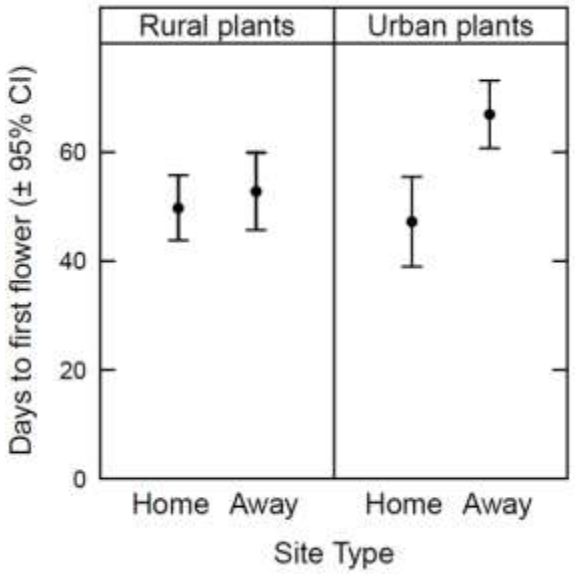

Figure 2.5 Effect of plant type (rural vs. urban seed origin) and planting location (home site vs. away site) on days until first flower. Urban plants took longer to produce flowers when planted in away sites (19.7 days ± 4.1 se, t-ratio = 4.76, $p < .0001$) while rural plants did not differ substantially in days to first flower between home and away sites (3.0 days ± 3.5 se, t-ratio = -0.87, $p = .3859$, see Table 2.1 for Type III F-tests).

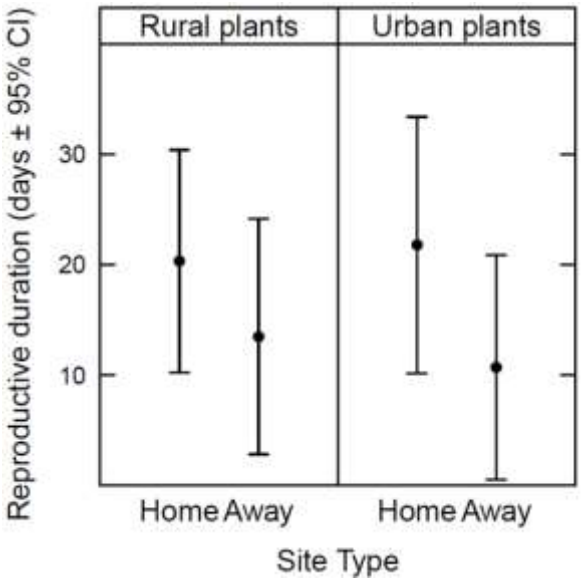

Figure 2.6 Effect of plant type (rural vs. urban seed origin) and planting location (home site vs. away site) on reproductive duration. Urban plants flowered roughly twice as long at home sites than at away sites (11.1 days longer ± 5.0, t-ratio = 2.23, p = .0291) while rural plants flowered roughly 50% longer at home (6.8 days longer ± 4.1 t-ratio = 1.64, p = .1058) (see Table 2.1 for Type III F-tests).

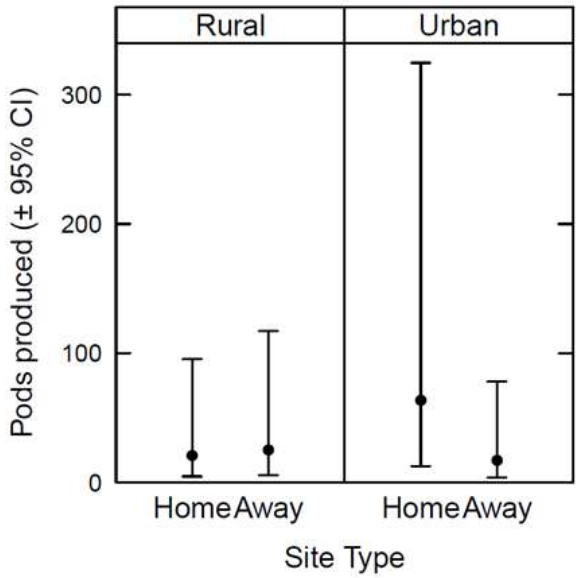

Figure 2.7 Effect of plant type (rural vs. urban seed origin) and planting location (home site vs. away site) on total seed pods produced. We log-transformed maximum pods prior to analysis - estimated marginal means presented here are back-transformed to the original response scale. Urban plants produced nearly four times as many seed pods at home sites compared to away sites (t-ratio = 2.04, p = .0448), whereas rural plants produced only 20% more seed pods at home sites than at away sites (t-ratio = -0.36, p = .7186). However note that Type III F-tests for the full model suggest marginal clarity for this result, suggesting further experimentation is needed to fully characterize this effect (see Table 2.1).

Table 2.1 Mixed Model Statistics for Effects of Plant Type and Home vs. Away on Fitness Components

Response variable	Effect	Sum Sq	NDF	DDF	F value	Pr(>F)
Survival duration	plant_type	79	1	161.79	0.1647	0.6854
	home_away	501	1	161.22	1.0374	0.31
	plant_type:home_away	41071	1	161.97	85.1267	2E-16
	site_pair (random)					
Days to first flower	plant_type	183.06	1	25.074	2.7542	0.10946
	home_away	1210.02	1	74.044	18.2053	5.8E-05
	plant_type:home_away	619.06	1	35.051	9.3141	0.00432
	plant_mom (random)					
	site_pair (random)					
Reproductive duration	plant_type	6.15	1	66.384	0.0699	0.79235
	home_away	1065.14	1	66.624	12.105	0.00089
	plant_type:home_away	28.37	1	51.603	0.3224	0.57261
	site_pair (random)					
Seed pods produced	plant_type	0.32911	1	67.137	1.1399	0.2895
	home_away	0.80947	1	67.486	2.8036	0.09868
	plant_type:home_away	0.70814	1	64.199	2.4527	0.12224
	site_pair (random)					

Note: Random effects were removed or retained based on likelihood ratio tests. Denominator degrees of freedom for fixed effects were calculated according to Satterthwaite's method. Seed pods produced were log-transformed prior to analysis.

2.5 Discussion

Urbanization has well-documented ecological and environmental changes, but the short-term and long-term impacts of evolutionary mechanisms still remain unclear across taxa (Johnson & Munshi-South, 2017). Insights from the fields of evolutionary biology and urban ecology have produced numerous predictions regarding how urban plant populations might evolve (Johnson et al., 2015). More urban field studies are needed, at different spatial

and temporal scales, that examine the relative roles of neutral and selective processes across plant taxa that differ in: 1) life history traits (e.g., annual, perennial, herbaceous, woody, self-pollinating, self-incompatible), 2) ecological strategies (e.g., stress-tolerant, stress-resistant), and 3) seed dispersal mechanisms and pollination syndromes (biotic and abiotic).

Table 2.2 Number of Seedlings Present in each Treatment Combination for each Mixed Model Analysis

Plant type	Site type	Survival duration	Days to first flower	Reproductive duration	Seedpods produced
Rural	home	40	29	25	26
Rural	away	49	14	13	13
Urban	home	42	9	7	7
Urban	away	37	28	28	28

Note: Number of seedlings differs because not all seedlings survived to each reproductive stage and because we planted additional seedlings at site four after the initial seedlings were destroyed immediately after planting.

We chose *Capsella bursa-pastoris* (shepherd's purse) as our study species by virtue of its worldwide distribution in cities and life history traits that suggest improved ability to adapt to urban abiotic stress. These traits include: 1) short generation times and abundant seed set (McDonnell & Hahs, 2015), 2) ruderal habit and occurrence in trampling-prone, urban settings (e.g., sidewalk cracks and roadsides) (McKinney, 2006; Vallet et al., 2010), 3) proclivity towards self-pollination, and 4) noted phenotypic variability, possibly due to tetraploidy (Neuffer et al., 2018). Previous work showing early and late flowering genotypes across latitudinal gradients (Neuffer et al., 2011) suggests potential for local adaptation across other transitional zones, particularly urban-rural gradients. Urban-rural gradient studies have long been used to study local adaptation of urban and rural

populations; such studies "provide an opportunity to explicitly examine the role of humans" and the human-built environment (McDonnell & Pickett, 1990).

We used a reciprocal transplant experimental approach that is considered the most direct method to test for local adaptation in plant populations (Franks et al., 2014). Few studies to date have used this methodology to quantify plant adaptation along urban-rural gradients (Gorton et al., 2018). The results of our reciprocal transplant experiment provide strong evidence that *C. bursa-pastoris* is adapting locally along the urban-rural gradient of New York City. One clear result that supports local adaption is reproductive duration, as both urban and rural plants produced flowers and seed pods about twice as long at home sites relative to away sites (Figure 2.6). Additionally, urban plants flowered earlier (Figure 2.5) and produced substantially more seed pods at urban home sites than at away rural sites on average in our experiment (Figure 2.7).

In terms of fitness, flowering time is a key life-history trait for ruderal species that live in disturbed environments (Toorop et al., 2012). Annual plants have a short window of time to complete their life cycle compared to perennials, particularly in disturbance-prone environments, and urban plants may trade-off in allocation towards growth in favor of reproduction as disturbance increases. A delay in flowering time allows for a longer period of vegetative growth, but it is a risky strategy for plants that live in very disturbed habitats (Ritland, 1983). Our lifespan results support this, as plants at rural sites lived nearly twice as long as plants in urban sites (Figure 2.4). Clearly, life in the city is short, and plants that go to flower and seed quickly have a fitness advantage in these environments.

An alternative hypothesis is that the significant delay in urban plant flowering at rural sites may indicate a release from stress factors (e.g., poor soils) typically found in

urban environments, thus facilitating a longer vegetative period for those urban populations when they are away. Ruderal plants living in nutrient poor soils often favor seed production over vegetative growth (Grime, 1977).

Capsella bursa-pastoris is a stress-tolerant ruderal plant (*S*-*R*), a strategy that lends itself to a life in urban environments (Grime, 1977). The species' global distribution in cities might suggest that it is preadapted to these environments (McDonnell & Hahs, 2015). However, a preadapted nature does not necessarily lead to persistence in these environments, and established populations may still be shaped by evolutionary mechanisms over time. Our hypothesis that urban abiotic stress leads to locally-adapted populations along an urban-rural gradient was supported by our reciprocal transplant experiment's results: both urban and rural plant types flowered earlier and longer at home compared to away. Reciprocal transplant experiments test for local adaptation (Franks et al., 2014), and urban plant fitness (e.g., seed pod number) was four times greater at home than away (Figure 2.7). Further analysis will be needed to establish a genetic basis for the observed differences in fitness (Franks et al., 2014). Given our experimental design, we also cannot rule out the role of epigenetics (e.g., maternal effects). But, if our observations are due to local adaptation of these populations, the key question is: which environmental factors are primarily responsible?

The most important environmental difference between our urban and rural sites is increased disturbance at urban sites. This is clear from our results that show that plants survived only half as long at urban sites compared to rural sites (Figure 2.4). We observed several mechanisms of disturbance in urban habitats that likely explain these shortened lifespans, including human foot traffic, animal traffic and excrement, vehicular traffic,

garbage litter, and various other human activities. However, in spite of this selective pressure to flower earlier, we did not find that plants from urban populations generally flowered earlier than rural populations (Figure 2.5). Instead, we found that once they did begin to flower, both plants from urban and rural populations flowered longer at home sites than at away sites (Figure 2.6). This suggests that if they survived long enough to begin flowering, something else about the growth environment at their home sites allowed them to be better adapted and to produce flowers for a longer duration.

One key way that urban areas differ from rural areas is that cities experience higher relative air temperatures (i.e., urban heat island effect) that may allow urban populations to germinate, produces leaves, and flower sooner than rural populations. However, cities also have colder winter ground temperatures than in adjacent rural areas (Thompson et al., 2016), and this effect could carry over into the spring when *C. bursa-pastoris* is most active. Temperature does influence flowering, so fluctuations could potentially lead to early or late-flowering genotypes over time (Neuffer et al., 2011; Neuffer & Eschner, 1995; Slotte, Holm, McIntyre, Lagercrantz, & Lascoux, 2007). If warmer air temperatures in cities affect flowering time in *C. bursa-pastoris*, we might expect urban plants to shift to an early-flowering ecotype over generations. Given this scenario, we would predict that urban plants would also flower earlier at away sites, which we did not observe. Alternatively, if *C. bursa-pastoris* flowering time is not regulated at the genetic level, indicating plasticity, we would predict rural plants to flower sooner in the warmer urban air conditions, which we also did not observe. If colder winter ground temperatures in cities play a larger regulatory role in the flowering time of this short, basal species, we might expect urban plants to shift to a late-flowering ecotype over generations. But, urban plants

flowered sooner in urban sites compared to away sites. Our temperature data suggest that our sites had similar air and ground temperatures during the experiment, likely due to relatively close spatial proximity. Therefore, we suspect another environmental factor is driving the longer reproductive duration we observed at home sites.

Pollution is another aspect to consider, as urban soils tend to have increased presence of salt and heavy metals (Del Tredici, 2007; Grimm et al., 2008). There is evidence that *C. bursa-pastoris* is a bio-indicator for heavy metals including lead, cadmium, zinc, and copper (Aksoy, Hale, & Dixon, 1999). However, our soil heavy metal analyses (See Table A.4) did not show any clear distinctions between rural and urban sites, likely due to small sample size and wide concentration ranges among sites. Arsenic, cadmium, chromium, manganese, and selenium occurred at effectively equivalent average levels. Urban sites had higher average lead, magnesium, and sodium. Rural sites had higher average aluminum, cobalt, copper, iron, and zinc. Some sites had metal concentrations well above all other sites. Soil from two rural sites and one urban site had average copper concentrations that exceed levels considered toxic to plants (60-125 μg/g dry weight) (Aksoy et al., 1999). Average zinc concentration was higher in rural soils, and soil from all urban and rural sites surpassed levels considered toxic to plants (70-400 μg/g dry weight) (Aksoy et. al, 1999). Lead levels exceeded the toxic range in all sites except for one rural site (100-400 μg/g dry weight) (Aksoy et al., 1999). Site four, the street tree pit located in Brooklyn, had an average lead concentration of 1780 μg/g dry weight, more than four times above the range considered toxic for plants. Arsenic values spiked at three sites within parks, one urban and two rural. Even though we did not see clear distinctions between

urban and rural heavy metal concentration, the variation at the site level may indicate that populations are locally adapted to the specific metal profile of their home site.

We hypothesize that local adaptation is driven by exposure to abiotic stress, however we must also consider biotic environmental factors that can influence fitness at home and away sites. For example, it is possible that plant-soil feedbacks could cause plants to have higher fitness at home sites than at away sites. If plant populations differentially support soil biota that are mutualistic, it could enhance plant growth within those populations and lead to increased fitness, whereas negative plant-soil feedbacks would reduce fitness (Wardle et al., 2004). However, we suspect our results are primarily due to abiotic factors for several reasons. Plant-soil feedbacks typically occur at the plant species level. Adaptation at our urban and rural sites would require localized coevolution between below-ground soil biota at the population level, and such coevolution has not been widely reported. Additionally, the majority of plant-soil feedback examples show negative effects, whereas our findings would require positive effects (Kulmatiski, Beard, Stevens, & Cobbold, 2008).

Our results suggest that the observed differences in the urban and rural populations are due to local adaptation. Additional studies are needed to identify 1) which environmental factors are selectively shaping urban populations and 2) how urban and rural populations differ in genetic response to these factors. To address the former, we conducted laboratory abiotic stress trials, and we present those results in Chapters 3 and 4. Future directions include 1) comparing genetic differences between populations using population genomics, 2) investigating the impact of specific genes on adaptation using gene expression

analysis, and 3) expanding our study to include other cities to determine whether our results are generalizable.

CHAPTER 3

LEAF TRAITS PREDICT GROWTH OF THE COSMOPOLITAN WEED *CAPSELLA BURSA-PASTORIS* UNDER DROUGHT CONDITIONS

3.1 Overview

In the previous study, we found evidence that populations of *Capsella bursa-pastoris* are locally adapting to urban environments in New York City. Here, we hypothesize that populations are being shaped by urban water stress regimes. High proportion of impervious surfaces in cities leads to frequent drought and periodic flooding, such that urban plant populations more frequently experience water stress compared to nearby rural populations.

We conducted a series of laboratory trials using plants (n = 392) representing urban and rural populations from the New York metropolitan area. If urban populations of *C. bursa-pastoris* are uniquely adapted to water stress, we expect urban plants to demonstrate 1) higher relative fitness compared to rural plants when exposed to water stress and 2) leaves that better mitigate the negative effects of water stress. We ran three trials, subjecting plants to different degrees of soil drying treatments including: drought, flood, cyclic drought and flood, and a well-watered control. We quantified five leaf traits among plants in the drought and control treatments in the third trial. Very few plants produced flowers or seed pods, and there was no mortality. Therefore, we used final plant size as a proxy for fitness. We quantified final plant size as basal area of surviving plants at the end of each trial, and found no consistent patterns across all three trials. Plants responded strongly to drought by increasing leaf dry matter content (LDMC), increasing stellate trichomes, and decreasing single-haired trichomes. Urban plants produced fewer stomata, while rural plants produced more in response to drought. Specific leaf area did not vary

among plant type nor water stress treatment. Leaf traits predicted final plant size in the drought treatment but not in the control. Wild plants endure much abiotic stress in urban areas, and it is difficult to pinpoint which factor plays the largest selective role for a given species. Additional experimentation will investigate the roles of other environmental stressors.

3.2 Background

Urbanization is an increasingly global phenomenon, but the evolutionary consequences of urban environments remain poorly understood. Efforts to understand how wild populations within and around cites evolve have accelerated over the last two decades (Cheptou et al., 2008; Cheptou et al., 2017; Donihue & Lambert, 2015; Dubois & Cheptou, 2017; Gorton et al., 2018; Grimm et al., 2008; Lambrecht et al., 2016; Thompson et al., 2016; Yakub & Tiffin, 2017). Much of this work is due to an established theoretical understanding of the ecological implications of urbanization (Aronson et al., 2015; Hobbs et al., 2009; Johnson & Munshi-South, 2017; McDonnell & Pickett, 1990). Existing and expanding urban areas bring about both gradual and abrupt environmental changes that disrupt ecosystems and their associated services (McDonnell & Hahs, 2015).

In particular, urbanization causes abrupt habitat destruction and gradual habitat degradation, creating powerful changes at different spatial and temporal scales. Compared to adjacent rural ecosystems, urban ecosystems experience characteristic changes in abiotic factors including increased air temperatures, air pollution, soil pollution from metals and de-icing salts, soil compaction, higher soil pH, limited soil volume, and altered nutrient, water, and disturbance regimes (Aronson et al., 2015; Del Tredici, 2007; McDonnell & Hahs, 2015; Sjöman & Nielsen 2010). These changes can cause great physiological stress

in urban populations, particularly in sessile organisms like plants that cannot quickly relocate to more favorable conditions. The degree of abiotic stress increases with the magnitude of urbanization, presenting researchers opportunities to examine how populations differ in response to urban-associated stress factors present along urban-rural gradients (McDonnell & Pickett, 1990).

We previously conducted a reciprocal transplant experiment with the weedy annual species *Capsella bursa-pastoris* (L.) Medik. (Brassicaceae) (shepherd's purse) in New York City. We found evidence that populations of *C. bursa-pastoris* are locally adapting to conditions in urban environments: urban plants demonstrated higher relative fitness (e.g., longer reproductive duration and seed pod production) compared to rural plants in urban sites and urban plants in rural sites (see Chapter 2). This supports the hypothesis that urban populations of *C. bursa-pastoris* have undergone adaptive evolution in response to abiotic stress in urban environments, but the driver of this selection remains unknown.

One possibility is that higher relative air temperature in urban sites (i.e., urban heat island effect) could cause populations in urban sites to acquire more resources sooner and begin flowering more quickly. Conversely, snow-removal efforts in cities result in colder winter ground temperatures compared to adjacent rural areas, where populations of short-statured plants remain blanketed with snow (Thompson et al., 2016). These altered temperature regimes could drive adaptive selection and result in early or late-flowering genotypes, respectively. However, we did not find evidence of conserved differences in flowering time among our rural and urban populations. For example, urban plants took much longer to mature (e.g., begin flowering) at rural sites compared to urban sites, but rural plants matured equivalently at both urban and rural sites. The latter result also

suggests we can rule out plasticity to temperature gradients as an explanation for our results.

Another possibility is that urban soil pollution is driving local adaption in urban populations. Urban soils tend to have higher concentrations of heavy metals and de-icing salts compared to rural soils (Del Tredici, 2007; Grimm et al., 2008). We performed soil tests during the reciprocal transplant experiment, and found that heavy metal concentration varied widely in both urban and rural soils (in collaboration with the Rodríguez-Freire Lab, NJIT). Urban soils had higher average concentrations for some heavy metals (e.g., lead, sodium, magnesium), whereas rural sites had higher average values for other metals (e.g., aluminum, cobalt, copper, iron, and zinc). However, the ranges of heavy metal concentrations was extremely varied across sites.

Altered disturbance regimes in urban areas are another potential driver shaping populations. Plant lifespan was twice as long in rural sites compared to urban sites during the reciprocal transplant experiment, due mostly to random disturbance events in urban sites. This increased disturbance could select for plants that reproduce quickly, and could explain why urban populations flowered sooner at urban sites compared to rural sites. However, delayed urban plant flowering at rural sites indicates that this early-flowering phenotype is not constitutive.

Urban water regimes may also be driving local adaptation in *C. bursa-pastoris*. Urban areas are often defined by high proportion impervious surface, and therefore this metric has been used to quantify the intensity of urbanization (Brabec, Schulte, & Richards, 2002). Impervious surfaces (e.g., roads, sidewalks, and buildings) prevent water infiltration into surface soils and alter water regimes, resulting in extreme moisture conditions. Urban

soils experience periodic flooding and frequent drought events due to proximity to and abundance of impervious surfaces in cities (Brabec et al., 2002). *Capsella bursa-pastoris* is commonly found in disturbed habitats like roadsides, sidewalk cracks, and street tree pits. It is short-statured (height 0.7 m) and the majority of leaves are produced as a basal rosette, making it particularly vulnerable to even mild flooding events. Urban heat island effect and compacted soils exacerbate the frequency of drought, which is the more common condition of urban soils (Just, Frank, & Dale, 2018).

Perhaps water stress is responsible for driving the local adaptation of *C. bursa-pastoris* populations that we observed in the field study along the urban-rural gradient of New York City. Water is the most limiting resource in plants, as it is the largest cellular component per volume in plant cells (Taiz, Zeiger, Møller, & Murphy, 2015). Local adaptation to water stress has been reported in wild populations of ruderal basal herbs in several common garden experiments. One study examined mountain populations of *Taraxacum officinale* (common dandelion) and found evidence that populations are locally adapting to dry alpine Andean environments (Molina-Montenegro, Quiroz, Torres-Diaz, & Atala, 2011). Another study tested populations of *Silene ciliata* (elegant catchfly) under different degrees of water stress in growth chambers and found that low altitude populations are adapting to moderate drought conditions, whereas extreme drought resulted in high rates of mortality in all populations (García-Fernández, Iriondo, Bartles, & Escudero, 2012). Greenhouse experiments of *Centaurea stoebe* (spotted knapweed) suggest that local adaptation to droughty environments helped facilitate the spread of this invasive species into new drought-prone ranges (Mráz, Tarbush, & Müller-Schärer, 2014).

Capsella bursa-pastoris has high variation in leaf morphology traits including rosette diameter, leaves per rosette, leaf area, leaf lobing, leaf thickness, epidermal cell thickness, and stomatal density (Neuffer et al., 2018). The species has been described as having at least four distinct basal rosette leaf types for over a hundred years (Shull, 1909), and more recent descriptions include those four and three other leaf-type classes (Iannetta et al., 2007) (Figure 3.1). The species has a nearly global distribution in cities, occurring everywhere except the wet tropics, indicating it has a large threshold for abiotic stress. Neuffer and colleagues (2018) subjected wild populations of *C. bursa-pastoris* to water stress trials and found that water-stressed plants grew leaves with denser mesophyll cells (which are heavily involved in photosynthesis) including two layers of palisade cells (involved in light absorption). They also found evidence that some *C. bursa-pastoris* leaf ecotypes (i.e., *heteris*, *rhomboidea*, and *simplex*) are more efficient in stomatal response to water stress compared to another ecotype (*tenuis*). In light stress experiments, they found that stomatal density varied both by treatment intensity and population origin, and that plants originating from hot, dry locales maintained higher photosynthetic rates under intense light.

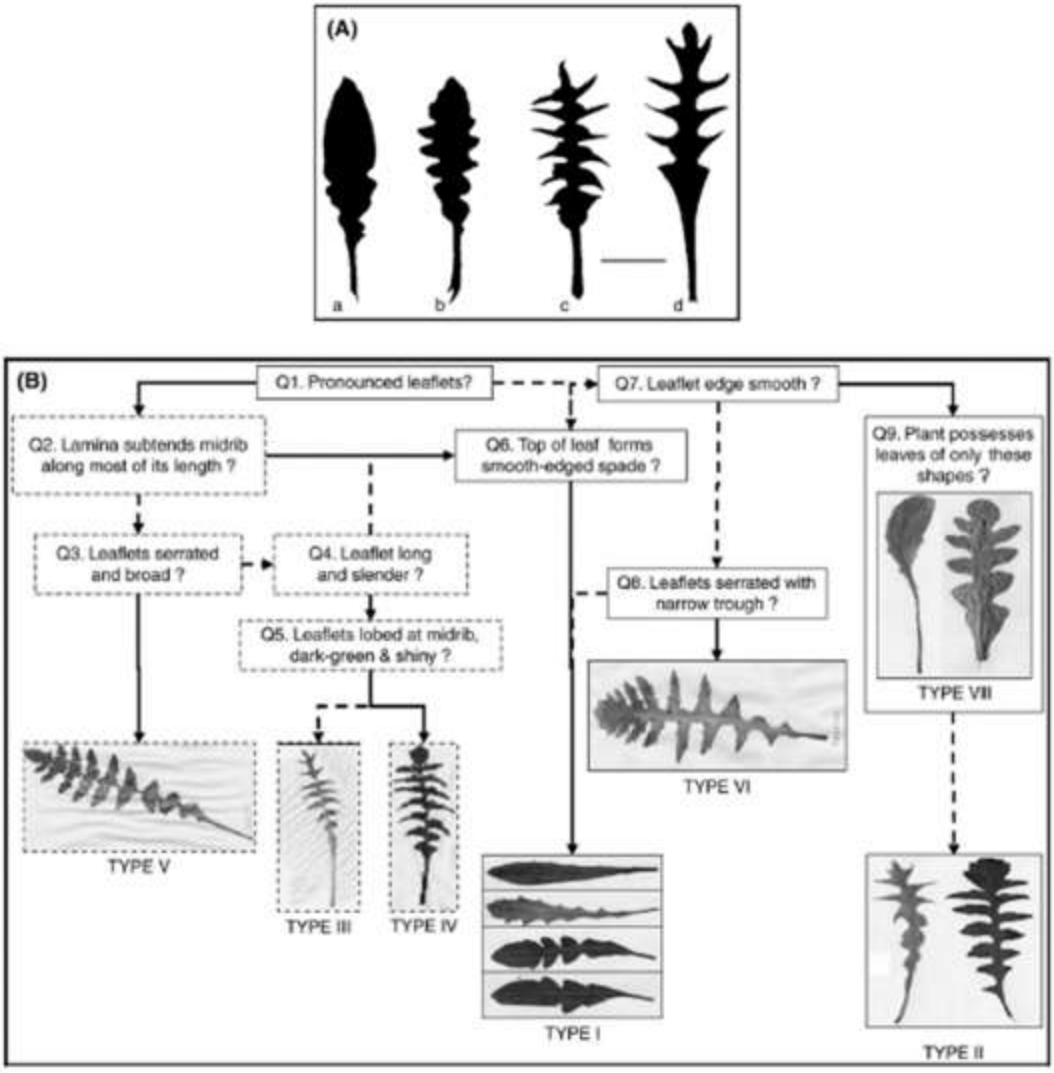

Figure 3.1 Variation in basal rosette leaf morphology of *C. bursa-pastoris* (from Iannetta et al., 2007). (A) The four leaf shapes originally described by Shull (1909): a, *simplex*; b, *rhomboidea*; c, *heteris*; and d, *tenuis*. Bar 10 mm. (B) Leaf key used to classify *C. bursa-pastoris* basal rosette leaves. Seven leaf shapes can be identified using the criteria described and the lines (solid = 'yes'; dashed = 'no').

The results of our reciprocal transplant experiment support the hypothesis that urban populations of *Capsella bursa-pastoris* are locally adapting to urban environments in the New York metropolitan area. We hypothesize that urban populations have undergone adaptive evolution in response to urban water stress regimes. To quantify the effect of water stress, we conducted a series of laboratory trials with plants from urban and rural

populations grown under different water regimes: control, drought, flood, and cyclic drought and flood. If urban populations are adapted to water stress, we expect that urban plants will 1) have higher relative fitness (e.g., reproductive duration and seed pod number) compared to rural plants in all stress treatments, 2) demonstrate higher tolerance to water stress (e.g., grow larger, have healthier leaves, live longer) compared to rural plants, and 3) have leaf phenotypes that better mitigate the effects of water stress (e.g., higher leaf dry matter content, lower specific leaf area, less stomata, more trichomes) compared to rural plants during drought treatments. We also predict that plants with drought-adapted leaf traits, be they from urban or rural populations, will perform better under drought conditions.

3.3 Materials and Methods

3.3.1 Seed Collection

Seeds collection surveys occurred May-June 2017 along an urban-rural gradient in the New York metropolitan area. We used a stratified random approach to select points along the gradient within 60-km from Times Square (40°75'N, 73°98'W). Surveyors followed local roads that spiraled out from each point until a roadside population of *Capsella bursa-pastoris* was located. We harvested seed pods from visibly healthy plants (n = 15; hereafter, "parent plants") at each population (n = 24). We used scissors to cut seed pod infructescences from the base of each plant and placed them into paper envelopes. We labeled the envelopes with population number, GPS coordinates, and parent plant voucher number. Envelopes were stored in a dry, well-ventilated location at the New Jersey Institute of Technology. We analyzed the local proportion impervious surface (IS) using QGIS (Version 2.18.3) (QGIS Development Team 2017) and 30 m resolution impervious surface

land cover data from the 2011 National Land Cover Database (Multi-Resolution Land Characteristics Consortium www.mrlc.gov/data accessed on 21 February 2017). The local impervious surface (0.0081 km^2) surrounding each population was used to designate populations as urban (IS_{local} > 70%) or rural (IS_{local} < 50%).

3.3.2 Seedling Rearing

In preparation for each of the three water stress trials, seeds were first geminated and seedlings reared as follows under uniform, non-water-stressed conditions. Seeds were sown into 4-pack seed-starting containers (806 Inserts, Grower's Solution, Cookeville, TN, USA) in standard (55 cm x 28 cm) plant trays on metal plant shelves (Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA). Trays were labeled with the date seeds were sown. We used a germination mix (LM-18 Germination Mix, Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA) and a 16 h photoperiod under fluorescent grow lights (Sun Blaze 44, ACF Greenhouses, Buffalo Junction, VA, USA). The germinated seedlings were kept under germination hoods and plant trays were rotated twice a week.

We repotted seedlings into 4x4 plastic pots (04.00 SQ TRAD TW M POT, Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA) when they were past the cotyledon stage and large enough to be handled without causing damage (~27 days post sowing). Each plastic pot was filled with the same volume of pre-moistened homogenized soil (Pro-Mix HP, Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA). Each seedling was gently removed from the 4-pack container, loose dirt carefully removed from roots, and roots dipped into a small water bath to remove as much propagation soil as possible without causing harm. We used a small plastic fork to carefully repot seedlings into their individual

4x4 pots. We labeled each seedling with vouchered plastic labels detailing seed collection population ID, parent plant ID, and seedling ID. At repotting, we recorded the following information: date sown, date repotted, seedling voucher, population, parent plant number, number of leaves, longest leaf, chlorotic leaves, damaged leaves, dead leaves, and the lengths of the primary and secondary axes of the basal rosette. The repotted seedlings were acclimated under germination hoods for one week and without hoods for another week. We tracked the temperature and humidity of the room (Humidity and Temperature Smart Home Environment System, AcuRite, Lake Geneva, WI, USA) and added humidifiers when needed to maintain an optimal growth environment. Water stress trials began after the acclimation period.

3.3.3 Water Stress Trials

We ran three water stress trials between November 2018 and December 2019. All trials combined contained 392 seedlings from 80 plant parents representing 10 urban populations and 9 rural populations. The trials involved different degrees of soil drying treatments (adapted from Osakabe et al., 2010 and Verslues, Agarwhal, Katiyar-Agarwhal, Zhu, & Zhu, 2006). The first and second trials included four water-stress treatments per experimental block: drought, flood, cyclic drought/flood, and control. The third trial included two water-stress treatments per experimental block: drought and control. We used room temperature water throughout all the trials, prestaging tap water in pre-cleaned 5-gallon opaque plastic buckets with lids. To begin the trial, all plants were watered to equal weight (300g) using room temperature water. Plants in the drought treatment had water withheld for one week, were partially watered (to 200g) at that time, and the process repeated. Plants in the flood treatment had soil submerged (>50%) for one week, allowed

to partially dry for one day, and the process repeated. Cyclic drought/flood plants had water withheld for one week, followed by soil submerged (>50%) for one week, and the process repeated. Control plants were kept evenly moist, and plants were watered twice a week (to 300g). Plant trays on shelves and plants within trays were rotated twice a week. All trials were exposed to a 16 h photoperiod under fluorescent grow lights (Sun Blaze 44, ACF Greenhouses, Buffalo Junction, VA, USA).

The first trial (November 16 2018 – December 26 2018) included 144 seedlings from 25 plant parents representing 3 urban and 3 rural populations. Each treatment block for the first trial contained 24 seedlings, with 6 seedlings per treatment. The second trial (April 9 2019 – August 1 2019) included 184 seedlings from 34 plant parents representing 5 urban and 4 rural populations. Most treatment blocks for the second trial contained 32 seedlings with 8 seedlings per treatment, except one block that contained 24 seedlings with 6 seedlings per treatment. The first and second trials each contained 6 treatment blocks of the four treatment types: control, drought, drought/flood, and control. When possible, the same parent plant was used for a given block, and different parent plants from the same population used in subsequent blocks.

The third trial (October 10 2019 – December 6 2019) included 64 seedlings from 31 plant parents representing 7 urban and 8 rural populations. Each treatment block (n = 4) contained 16 seedlings, with 8 seedlings per treatment. Here, populations were represented by two parent plants per treatment within each treatment block.

We recorded the following fitness data throughout the trials: alive or dead, longest leaf, number of leaves, number of dead leaves, number of chlorotic leaves, number of curled leaves, lengths of the primary and secondary axes of the basal rosette, number of

flowers, number of seed pods, number of primary flower and secondary flower stalks, number of open seed pods, and number of stunted seed pods. Fitness data were collected on days 0, 3, 6, 10, 15, 21, 28, and 40 during the first trial for a total of 6 weeks. We took fitness data on day zero and then weekly during the second and third trials. The second and third trials ran 15 weeks and 7 weeks, respectively. We quantified final plant size as basal area of living plants at the end of each trial.

3.3.4 Leaf Trait Measurements

Leaves were harvested from living plants at the end of the third trial (t =7 weeks) to perform leaf trait analyses. Plants were watered to 200g on the day before leaves were harvested to ensure turgor. Leaves were taken from plants (n = 50) that still bore at least three live, healthy, non-chlorotic leaves. Leaves were removed at the base of the petiole using scissors. The longest leaf was used to calculate leaf dry matter content and specific leaf area following methods described in Cornelissen et al., (2003). We used the second longest leaf to quantify stomatal density, and the third longest leaf to record trichrome density and morphology.

3.3.5 Leaf Dry Matter Content and Specific Leaf Area

Each longest leaf (n = 50) was weighed (PB4002-S Classic Plus scale, Mettler Toledo, Columbus, OH) immediately after it was removed from a plant. After weighing, we photographed the leaf (iPhone 5s, Apple Computer, Inc., Cupertino, CA) inside of a 10 cm x15 cm frame labeled with the seedling voucher number. We put the leaf into a small paper envelope, labeled the envelope with the seedling voucher number, and placed the envelope

into an 60ºC oven to dry for at least 72 h or until constant mass was achieved. We calculated leaf dry matter content (LDMC, mg g^{-1}) as:

$$\text{LDMC} = (\text{oven-dry mass}) / (\text{fresh mass}) \quad (3.1)$$

Leaf area was determined using image analysis (ImageJ 1.47) (Schneider et al., 2012). The measurement scale was set by drawing a line on the frame demarking a specific length (10 cm). The image was converted to grayscale (Image > Type > 8-bit) and made binary (Process > Binary > Make Binary). We analyzed the area of the leaf using the "*analyze particles*" command. We calculated specific leaf area (SLA, mm^2 mg^{-1}) as:

$$\text{SLA} = (\text{fresh leaf area}) / (\text{oven-dry mass}) \quad (3.2)$$

3.3.6 Stomatal Density

The lower epidermal surface of each second longest leaf (n = 50) was painted with clear nail polish immediately after it was removed from the plant. The painted leaves were allowed to air dry for at least 72 h. The epidermal peels were removed using clear transparent tape, and the tape was mounted onto microscope slides labeled with the seedling voucher number (adapted from Franks et al. 2009). Each microscope slide was viewed at 50X using a compound microscope (Nikon Eclipse Ts2, Nikon Inc., Minato City, Tokyo, Japan). All stomata in the field of view were counted at three stratified points on the leaf (e.g., lower leaf, middle leaf, upper leaf) to obtain average stomata density per leaf.

Stomata that were only partially in the field of view were counted if on the bottom and right and disregarded if on top and left of the field of view.

3.3.7 Stellate and Single-haired Trichome Density

We used the upper epidermal surface of each third longest leaf (n = 49) to quantify the density of stellate and single-haired trichomes (Figure 3.2) using a digital dissecting microscope (Nikon SMZ-25, Nikon Inc., Minato City, Tokyo, Japan) and associated software (NIS Elements 4.30, Nikon Inc., Minato City, Tokyo, Japan). We placed each leaf on the specimen stage and gently flattened it using a microscope slide. We drew a box (5.5 mm x 2 mm) at three stratified points on the leaf (e.g., lower leaf, middle leaf, upper leaf) and counted all single-haired and multi-branched (stellate) trichomes within the box to obtain average trichome density per leaf.

3.3.8 Statistical Analyses

Our experimental design included random effects for seed collection population, parental plant, and treatment block. We used a mixed model analysis using the R package lme4 (Bates et al., 2014) in R (R software v.3.5.1, R Core Team, 2019). For each trial, we fitted a full model including plant type, treatment type, and their interactions as fixed effects, and population, treatment block, and parent plant as random effects. We reduced the full models by individually removing the random effects, and used AIC (Akaike, 1974) to select the best fitting model for each trial. We performed F-tests for fixed effects using the Satterthwaite approximation for denominator degrees of freedom (Satterthwaite, 1946) using the R package lmerTest (Kuznetsova et al., 2017).

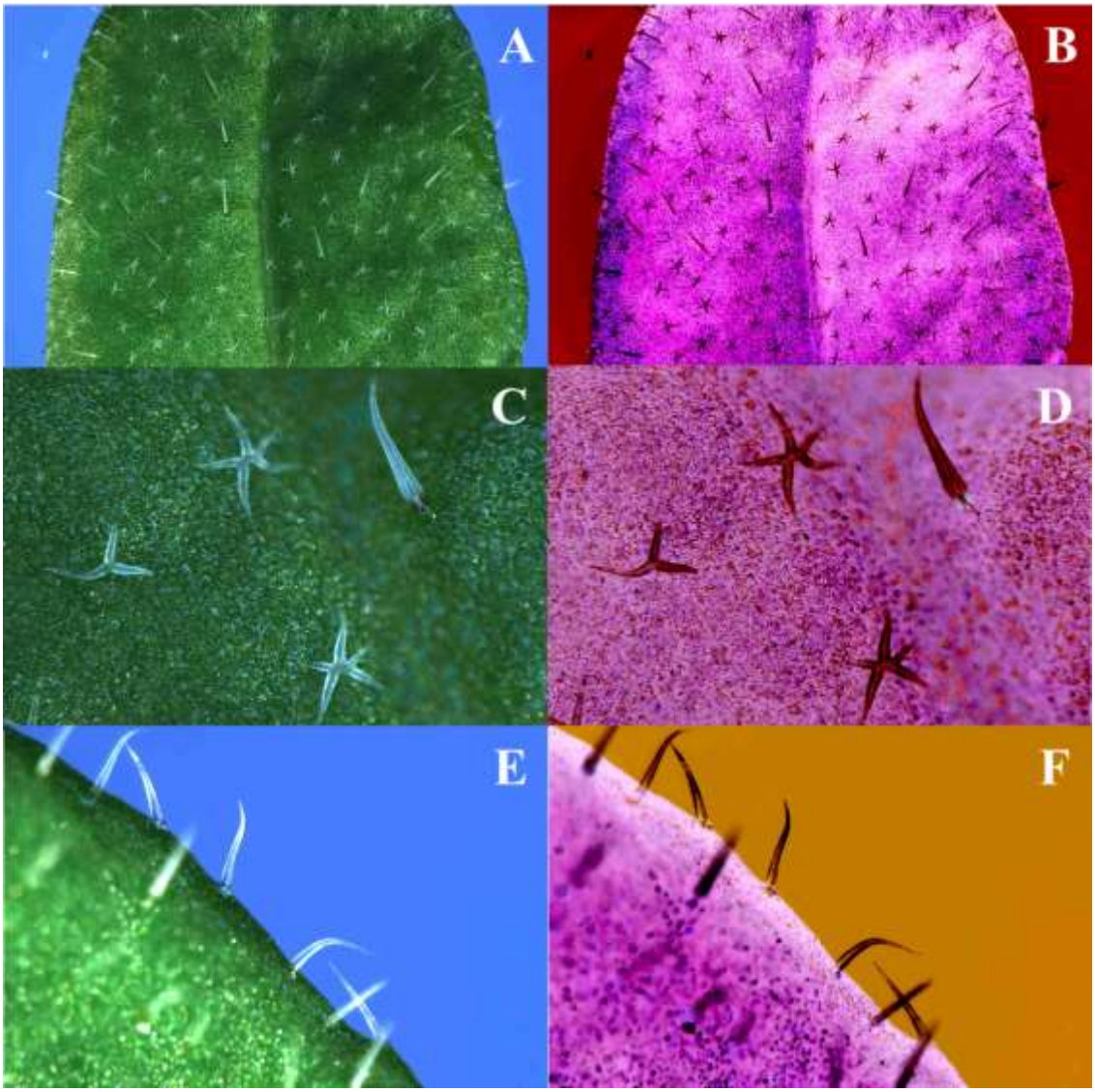

Figure 3.2 Leaf trichomes on *C. bursa-pastoris*. (A) Example upper epidermis showing single-haired and stellate trichomes and (B) inverted and contrasted. This leaf is from population 4 parental plant 59 (0.63X). (C) Detail of mostly stellate trichomes and (D) inverted and contrasted. This leaf is from population 13 parental plant 187 (8.6X). (E) Detail of single-haired trichomes and (F) inverted and contrasted. This leaf is from population 13 parent plant 186 (6X). Photos by Rebecca Panko.

We also conducted orthogonal contrasts (Littell, Milliken, Stroup, & Wolfinger, 1996; Sokal & Rohlf, 1995) using the R package emmeans (Lenth, 2019) with Satterthwaite denominator degrees of freedom to explicitly compare responses of each plant type to each

treatment. Lastly, we extracted estimated marginal means (Figures 3.3-3.5) using the R package emmeans (Lenth, 2019) for presentation in figures. When necessary, we log-transformed response variables to meet assumptions of normality and homoscedasticity. We back-transformed the estimated marginal means for presentation in figures.

To examine whether leaf traits predict final plant size under control and drought conditions, we performed principle components analysis (PCA) using prcomp from the R Stats Package (R Core Team and contributors worldwide, 2019) on our five traits, as some traits were strongly correlated with each other. As we wanted to use the PCA axes to predict final plant size, and because the drought and control treatments strongly affected the leaf traits, we ran the PCA on both the drought leaf trait data and the control leaf trait data separately. We used two separate mixed models (described above) to test for the effects of leaf traits on final plant size, one for each of the control and drought treatments.

3.4 Results

3.4.1 Water Stress Trials: Mortality and Fecundity

There was no plant mortality during any of the trials. Many seedlings from one rural population (population 23) began flowering before trials began, and therefore we do not include any plants (n = 28) from that population in our analyses here. Very few other plants produced flowers (12.6% across all trials). Among the three trials, 22% of plants flowered (26/120) in trial one, 7.6% of plants (14/184) in trial two, and 10% of plants (6/60) in trial three. In trial one, plants flowered in each treatment, all of which were urban plants except for one rural plant that flowered in the drought/flood treatment (See Appendix B, Figure B.1). Conversely, the majority of plants that flowered in trial two were rural, the exception

being two urban plants that flowered in the flood treatment. No plants flowered in the drought treatment of trial two (Figure B.1). Trial three had two urban plants and one rural plant produce flowers in both the control and drought treatments (Figure B.1). All plants that flowered in trials two and three produced seed pods, as did the majority (88.5%) of flowering plants in trial one when trials ended (Figure B.2).

3.4.2 Water Stress Trials: Final Plant Size

Since there was no mortality and fecundity was low, we used final plant size as a proxy for fitness (Younginger et al., 2017). Statistical analysis comparing final plant size for the first trial indicated that the best fitting model included only the random effect for seed collection population. Type III F-tests showed a significant interaction effect between treatment and plant type ($p < .05$, Table 3.1). Rural plants grew substantially and significantly less in response to drought compared to controls (-11776 ± 4695 mm^2; $t_{1,107.99} = -2.508$, $p < .05$), while flood and drought/flood had no effect (Figure 3.3). Urban plants were unaffected by any of the water stress treatments (Figure 3.3). Rural plants generally grew larger than urban plants in control (8483 ± 5556 mm^2; $t_{1,9.54} = 1.527$, $p=.1293$), flood ($+19540 \pm 5556$ mm^2; $t_{1,9.54} = 3.517$, $p < .01$), and drought/flood ($+13562 \pm 5556$ mm^2; $t_{1,9.54} = 2.441$, $p <.05$), whereas rural and urban plants had similar final plant sizes when grown in drought ($+973 \pm 5596$ mm^2; $t_{1,9.8} = 0.174$, $p = .8655$) (Figure 3.3).

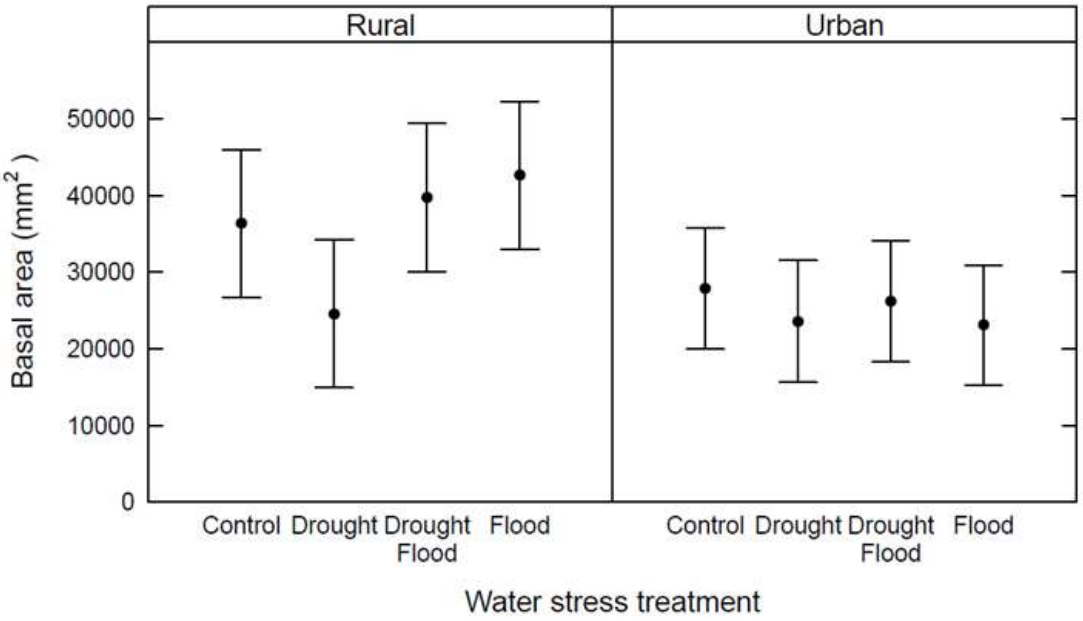

Figure 3.3 Effect of plant type (rural vs. urban) and treatment (control, drought, drought/flood, and flood) on final plant size measured as basal leaf area (mm^2) in the first trial. Error bars are 95% CI. Type III F-tests showed a significant interaction effect between treatment and plant type ($p < .05$) (Table 3.1). Control rural plants were slightly smaller than plants in the flood (-6279 ± 4695 mm^2; $t_{1,107.99} = -1.337$, $p = .184$) and drought/flood treatments (-3421 ± 4695 mm^2; $t_{1,107.99} = -0.729$, $p = .4678$), whereas controls were significantly larger (+11776 ± 4695 mm^2; $t_{1,107.99} = 2.508$, $p < .05$%) than plants grown in drought. Control urban plants tended to be larger than urban plants grown in both flood (+4779 ± 3834 mm^2; $t_{1,107.99} = 1.247$, $p = .2152$) and drought treatments (+4267 ± 3891 mm^2; $t_{1,108.04} = 1.097$, $p = .2752$), but were similar in size to urban plants grown in drought/flood treatments (+1658 ± 3834 mm^2; $t_{1,107.99} = 0.433$, $p = .6662$). Rural plants and urban plants in the first trial had similar final plant sizes when grown in drought (+973 ± 5596 mm^2; $t_{1,9.8} = 0.174$, $p = .8655$), whereas rural plants had much larger plants in the flood (+19540 ± 5556 mm^2; $t_{1,9.54} = 3.517$, $p < .01$) and drought/flood treatments (+13562 ± 5556 mm^2; $t_{1,9.54} = 2.441$, $p < .05$).

Table 3.1 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Water Stress Treatment and Plant Type on Final Plant Size in the First Trial

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Treatment	1569675965	523225322	3	108.004	3.9557	0.01016
plant_type	874653811	874653811	1	2.987	6.6126	0.08275
treatment:plant_type	1322255940	440751980	3	108.004	3.3322	0.02225

Note: final model included seed collection population as a random effect.

Statistical analysis of final plant size for the second trial indicated that the best fitting model included seed collection population and treatment block as random effects. Type III F-tests showed a substantial treatment effect ($p < .0001$, Table 3.2) (Figure 3.4), though rural plants did tend to be larger in drought treatments compared to control treatments ($+2869 \pm 1277$ mm^2; $t_{1,164.7} = +2.246$, $p < .05$) and larger than urban plants in drought treatments ($+3020 \pm 1722$ mm^2; $t_{1,19.8} = 1.754$, $p = .0949$) (Figure 3.4).

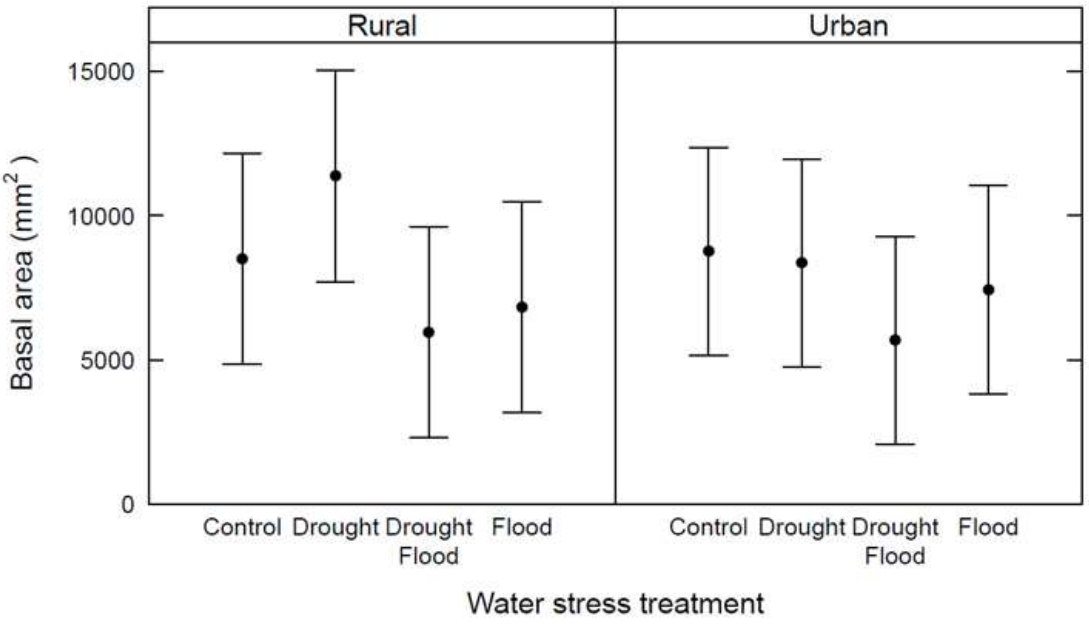

Figure 3.4 Effect of plant type (rural vs. urban) and treatment (control, drought, drought/flood, and flood) on final plant size measured as basal leaf area (mm^2) in the second trial. Error bars are 95% CI. Type III F-tests showed a substantial treatment effect ($p < .0001$) (Table 3.2), though rural plants did tend to be larger in drought treatments compared to control treatments ($+2869 \pm 1277$ mm^2; $t_{1,164.7} = +2.246$, $p < .05$) and larger than urban plants in drought treatments ($+3020 \pm 1722$ mm^2; $t_{1,19.8} = 1.754$, $p = .0949$).

Table 3.2 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Water Stress Treatment and Plant Type on Final Plant Size in the Second Trial

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatment	429837158	143279053	3	164.768	7.6384	<.0001
plant_type	4087247	4087247	1	6.973	0.2179	0.6549
treatment:plant_type	93219131	31073044	3	164.768	1.6566	0.1784

Note: final model included treatment block and seed collection population as random effects.

Response variables for the third trial were log-transformed to meet assumptions of normality and homoscedasticity. The best fitting model for the third trial included only treatment block for random effects. Type III F-tests showed no significant effects (Table 3.3). Variables were back-transformed for visualization, showing that final plant sizes are similar for both plant types in both treatments, though there is a trend towards smaller plant size under drought conditions (Figure 3.5).

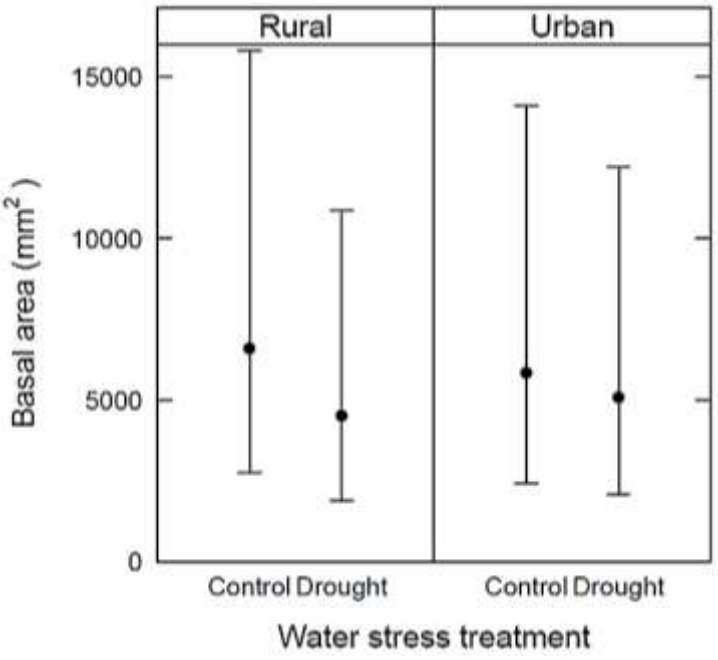

Figure 3.5 Effect of plant type (rural vs. urban) and treatment (control, drought) on final plant size measured as basal leaf area (mm^2) in the third trial. Error bars are 95% CI. Response variables for the third trial were log-transformed because they were log-normally distributed and to meet assumptions of normality and homoscedasticity. Variables were back-transformed for visualization. Type III F-tests showed no significant effects (Table 3.3).

Table 3.3 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Water Stress Treatment and Plant Type on Final Plant Size in the Third Trial

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatment	0.99752	0.99752	1	52.029	2.8975	0.09469
plant_type	0.00025	0.00025	1	52.151	0.0007	0.97841
treatment:plant_type	0.20337	0.20337	1	52.029	0.5907	0.44561

Note: final model included treatment block as a random effect.

3.4.3 Leaf Dry Matter Content

Statistical analysis of LDMC indicated that the best fitting model included only treatment block for random effects. Type III F-tests showed a significant treatment effect ($p < .05$, Table 3.4). Compared to controls, drought plants had more LDMC whether they were rural plants ($+27.286 \pm 12.5$ mg g^{-1}; $t_{1,43.1} = +2.176$, $p < .05$) or urban plants ($+19.339 \pm 12.6$ mg g^{-1}; $t_{1,43.5} = +1.536$, $p = .1317$) (Figure 3.6).

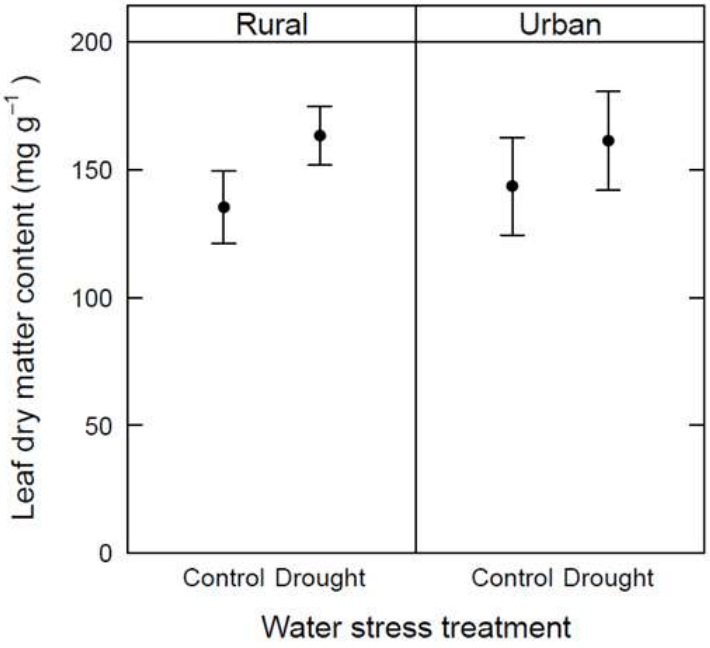

Figure 3.6 Effect of plant type (rural vs. urban) and treatment (control, drought) on LDMC (mg g^{-1}). Error bars are 95% CI. Type III F-tests showed a significant treatment effect ($p < .05$, Table 3.4). Compared to controls, drought plants had more LDMC whether they were rural plants ($+27.286 \pm 12.5$ mg g^{-1}; $t_{1,43.1} = +2.176$, $p < .05$) or urban plants ($+19.339 \pm 12.6$ mg g^{-1}; $t_{1,43.5} = +1.536$, $p = .1317$).

Table 3.4 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Water Stress Treatment and Plant Type on Leaf Dry Matter Content

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatment	6751.2	6751.2	1	43.262	6.8902	0.01193
plant_type	214.1	214.1	1	44.608	0.2185	0.64247
treatment:plant_type	195.9	195.9	1	43.317	0.1999	0.65699

Note: final model included treatment block as a random effect.

3.4.4 Specific Leaf Area

Statistical analysis of SLA indicated that the best fitting model included only treatment block for random effects. Type III F-tests showed no significant effects of treatment or plant type on SLA (Table 3.5), which varied little across all groups (Figure 3.7).

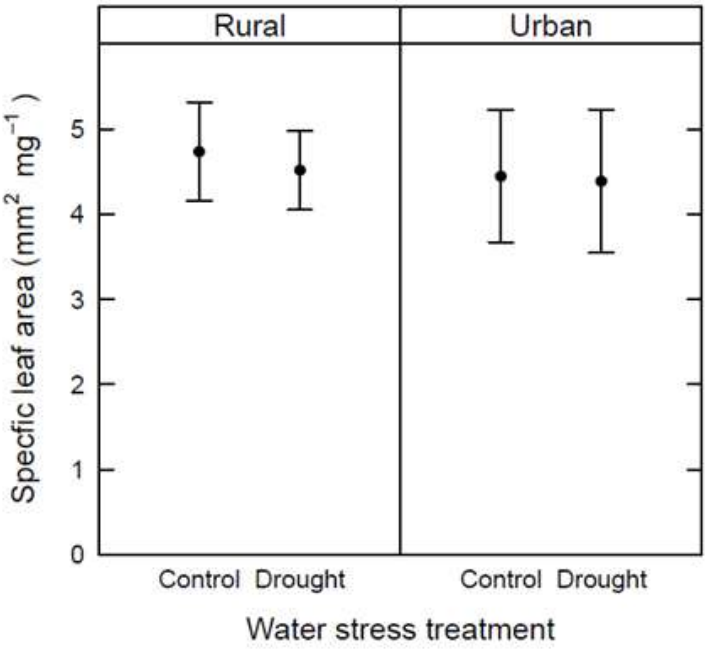

Figure 3.7 Effect of plant type (rural vs. urban) and treatment (control, drought) on SLA (mm^2 mg^{-1}). Error bars are 95% CI. Type III F-tests showed no effect for SLA, which was equivalent for all plants (Table 3.5).

Table 3.5 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Water Stress Treatment and Plant Type on Specific Leaf Area

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatment	0.39919	0.39919	1	43.115	0.2732	0.6039
plant_type	1.20164	1.20164	1	43.812	0.8224	0.3694
treatment:plant_type	0.00077	0.00077	1	43.137	0.0005	0.9818

Note: final model included treatment block as a random effect.

3.4.5 Stomatal Density

The best fitting model for stomatal density included parent plant as a random effect. Type III F-tests showed a significant interaction effect between treatment and plant type ($p < .05$, Table 3.6). Rural plants in drought produced more stomata ($+4.86 \pm 2.91$ cm^2; $t_{1,22.8} = +1.671$, $p = 0.1083$) compared to control plants, while urban plants in drought produced fewer stomata (-5.06 ± 2.95 cm^2; $t_{1,24.6} = -1.719$, $p = .0982$) compared to controls. Urban plants grown in drought produced significantly fewer stomata (-7.44 ± 3.43 cm^2; $t_{1,42.7} = -2.168$, $p < .05$) compared to rural plants (Figure 3.8).

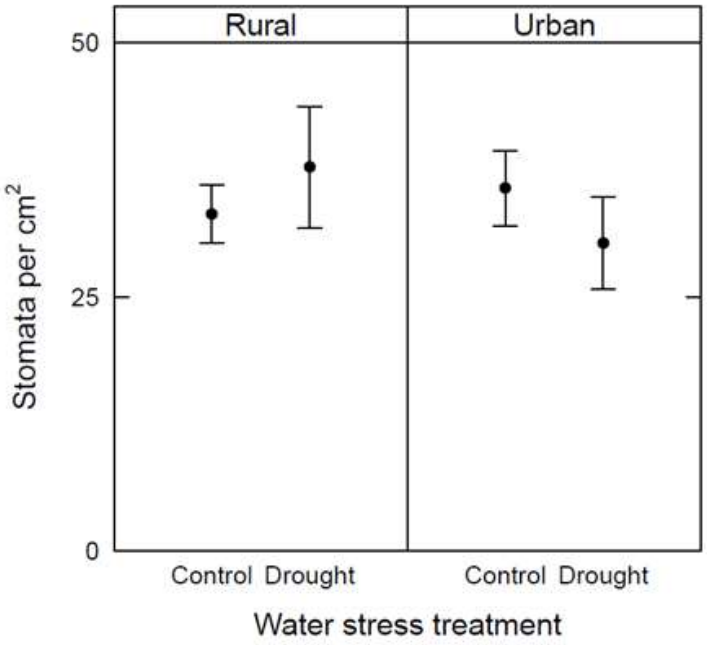

Figure 3.8 Effect of plant type (rural vs. urban) and treatment (control, drought) on stomatal density (cm^{-2}). Error bars are 95% CI. Type III F-tests showed a significant interaction effect between treatment and plant type ($p < .05$, Table 3.6). Rural plants in drought produced more stomata ($+4.86 \pm 2.91$ cm^2; $t_{1,22.8} = +1.671$, $p = 0.1083$) compared to control plants, while urban plants in drought produced fewer (-5.06 ± 2.95 cm^2; $t_{1,24.6} = -1.719$, $p = .0982$) compared to controls. Urban plants grown in drought produced significantly fewer stomata (-7.44 ± 3.43 cm^2; $t_{1,42.7} = -2.168$, $p < .05$) compared to rural plants.

Table 3.6 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Water Stress Treatment and Plant Type on Stomatal Density

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatment	0.128	0.128	1	23.663	0.0025	0.9608
plant_type	40.358	40.358	1	24.952	0.7753	0.3870
treatment:plant_type	299.198	299.198	1	23.663	5.748	0.0247

Note: final model included plant parent as a random effect.

3.4.6 Stellate and Single-haired Trichome Density

One of the 50 leaves used to quantify trichome density was not flat enough to accurately perform trichome counts. The best fitting models for both single-haired and stellate trichome density included parental plant as a random effect. Type III F-tests showed significant treatment effects in both cases ($p < .0001$, Table 3.7, and $p < .01$, Table 3.8, respectively). Both plant types produced fewer single-haired trichomes under drought conditions compared to controls (rural: -46.064 ± 17.7 hairs cm^{-2}; $t_{1,23.6} = -2.597$, $p < .05$; urban: -75.167 ± 17.4 hairs cm^{-2}; $t_{1,24.6} = -4.328$, $p < .001$) (Figure 3.9). Conversely, both plant types produced more stellate trichomes under drought conditions compared to controls (rural: $+103.29 \pm 35.6$ hairs cm^{-2}; $t_{1,23.2} = +2.902$, $p < .01$; urban: $+67.06 \pm 34.8$ hairs cm^{-2}; $t_{1,24.1} = +1.925$, $p = .0661$) (Figure 3.10).

3.4.7 Leaf Traits and Plant Size

Leaf traits were substantially correlated, with the first PCA axes explaining 40 and 48% of the trait variation among the control and drought plants, respectively (Figures 3.11 and 3.12). Trait loadings on the first PCA axis were driven primarily by SLA and LDMC, whereas the loadings on the second axis were single hairs and stomatal density (Figures 3.11 and 3.12). The best fitting models for leaf traits and final plant size for both control

and drought included treatment block as a random effect. Type III F-tests found no effect for control plants (Table 3.9), whereas leaf trait PC1 values predicted final plant size of drought plants ($p < .05$, Table 3.10) (Figure 3.13).

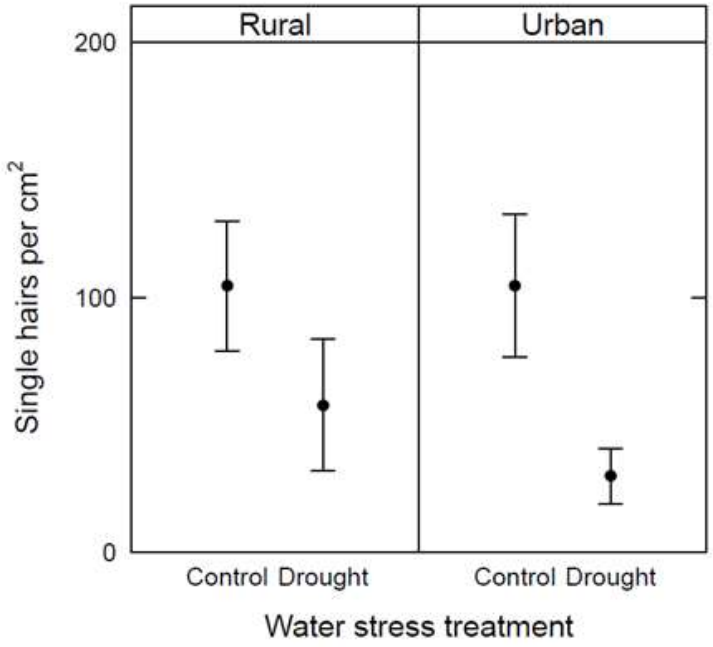

Figure 3.9 Effect of plant type (rural vs. urban) and treatment (control, drought) on single-haired trichomes. Error bars are 95% CI. Type III F-tests showed a significant treatment effect ($p < .0001$, Table 3.7). Both plant types produced fewer single-haired trichomes under drought conditions compared to controls (rural: -46.064 ± 17.7 hairs cm^{-2}; $t_{1,23.6} = -2.597$, $p < .05$; urban: -75.167 ± 17.4 hairs cm^{-2}; $t_{1,24.6} = -4.328$, $p < .001$). Rural plants tended to produce more single-haired trichomes compared to urban plants in drought conditions ($+28.340 \pm 18$ hairs cm^{-2}; $t_{1,44.6} = +1.577$, $p = .1218$).

Table 3.7 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Water Stress Treatment and Plant Type on Single-haired Trichome Density

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatment	44272	44272	1	24.067	23.8523	<.0001
plant_type	1858	1858	1	24.691	1.0008	0.3268
treatment:plant_type	2551	2551	1	24.067	1.3746	0.2525

Note: final model included plant parent as a random effect.

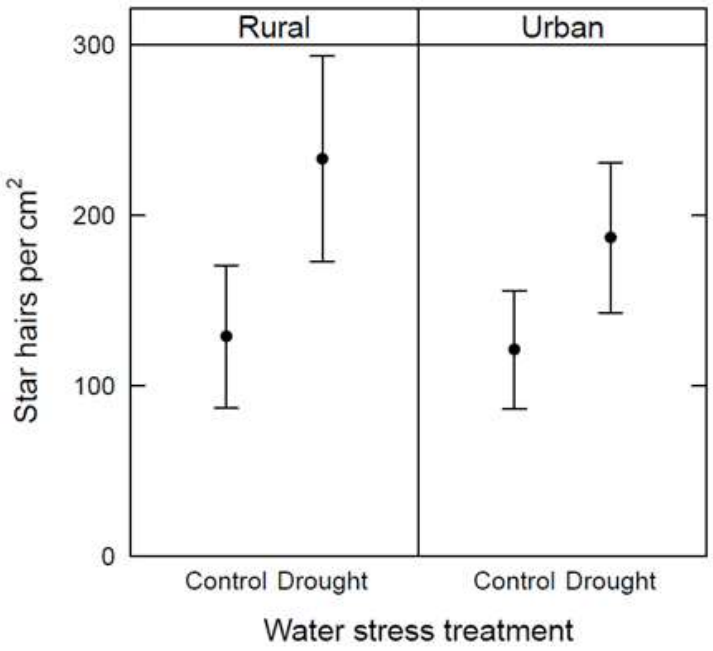

Figure 3.10 Effect of plant type (rural vs. urban) and treatment (control, drought) on stellate trichomes. Error bars are 95% CI. Type III F-tests showed a significant treatment effect ($p < .01$, Table 3.8). Both plant types produced more stellate trichomes under drought conditions compared to controls (rural: $+103.29 \pm 35.6$ hairs cm^{-2}; $t_{1,23.2} = +2.902$, $p < .01$; urban: $+67.06 \pm 34.8$ hairs cm^{-2}; $t_{1,24.1} = +1.925$, $p = .0661$). Rural plants tended to produce slightly more stellate trichomes under drought conditions compared to urban plants ($+45.32 \pm 35.8$ hairs cm^{-2}; $t_{1,44.7} = +1.266$, $p = .2120$).

Table 3.8 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Water Stress Treatment and Plant Type on Stellate Trichome Density

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatment	87548	87548	1	23.66	11.6979	0.002275
plant_type	7433	7433	1	24.21	0.9932	0.328812
treatment:plant_type	3960	3960	1	23.66	0.5291	0.474137

Note: final model included parent plant as a random effect.

3.5 Discussion

Water stress causes ionic imbalances in plants, which lead to cellular responses including cell dehydration, reduced water potential (ψ), and hydraulic resistance in vascular tissues (Taiz et al., 2015). Initial response to water stress involves stomatal closure to reduce

transpiration and conserve limited water within plant cells. This action effectively reduces water loss, but it also inhibits photosynthesis. Reduced biological activity during periods of water stress, a strategy called drought evasion, might allow plants to survive unfavorable conditions (Larcher, 2003; Santiso & Retuerto, 2017). But reduced photosynthetic rate results in secondary effects including decreased leaf area, smaller plant sizes, slower metabolic rates, leaf abscission, ion toxicity, and cell death (Taiz et al., 2015).

Table 3.9 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Leaf Trait PC1 on Final Plant Size for Plants Grown Under Control Conditions

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
PC1	5311	5311	1	19.805	0.0003	0.986
PC2	1469743	1469743	1	19.45	0.0874	0.7707

Note: final model included treatment block as a random effect.

Table 3.10 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Leaf Trait PC1 on Final Plant Size for Plants Grown Under Drought Conditions

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
PC1	10395457	10395457	1	20.202	5.4928	0.02943
PC2	2391235	2391235	1	20.259	1.2635	0.27413

Note: final model included treatment block as a random effect.

Short-lived annual species may experience dramatic shifts in development in extreme water-stressed environments. These plants have two distinct life cycle phases: a period of vegetative growth to acquire resources and a reproductive phase (Brachi, Aimé, Glorieux, Cuguen, & Roux, 2012). Since annual plants have a single growing season to complete both phases, they are particularly sensitive to extreme conditions. During severe water stress, annual plants may shift immediately to the reproductive phase, producing flowers before the plant has reached mature size. A smaller plant size results in less photosynthetic ability even if water stress is removed. This can result in fewer and smaller

seeds (Taiz et al., 2015), a maternal effect that could influence the fitness of the resulting offspring (Krannitz, Aarssen, & Dow, 1991).

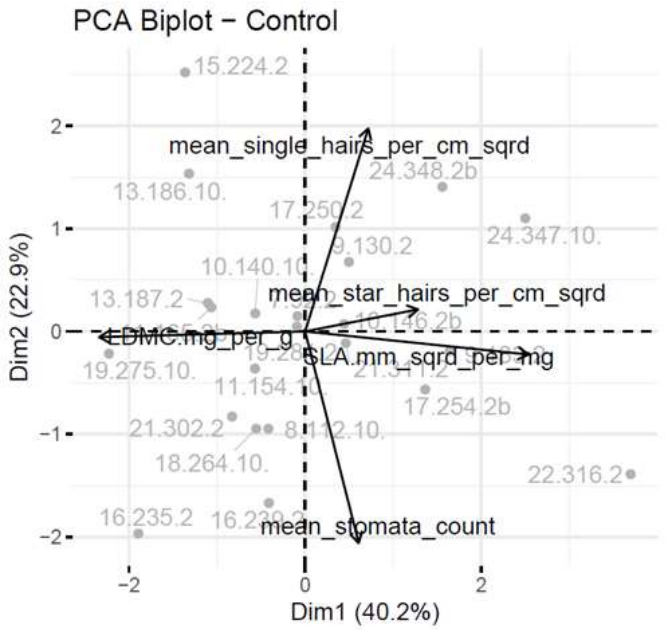

Figure 3.11 PCA of leaf traits for control plants.

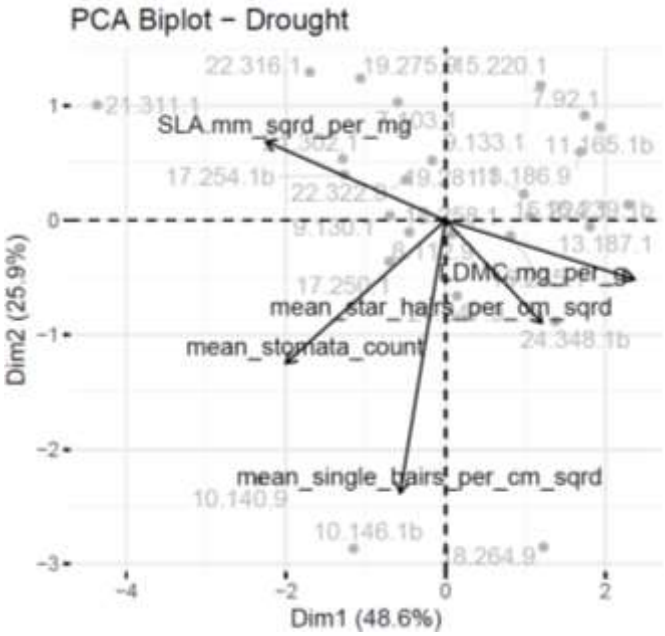

Figure 3.12 PCA of leaf traits for drought plants.

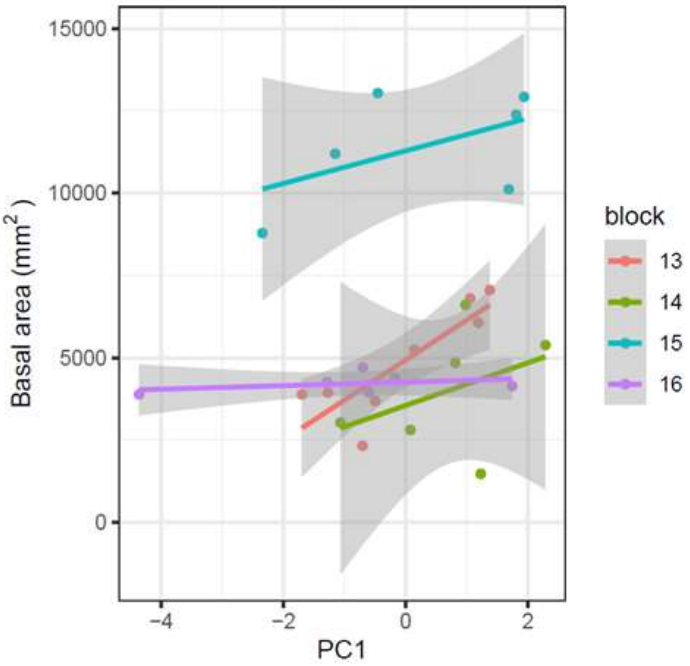

Figure 3.13 Type III F-tests showed that leaf trait PC1 values of Figure 12 predict the final plant size of drought plants ($p < 0.05$).

However, the physiological response to water stress does not necessarily always lead to a shift in developmental timing in annual species. In addition to drought evasion, plants also employ desiccation tolerance and desiccation avoidance (Larcher, 2003; Santiso & Retuerto, 2017). Plants that demonstrate desiccation tolerance and avoidance function during drought conditions due to tissues that sequester water or prevent water loss. Water stress response in plants is complex (Singh & Laxmi, 2015), and plants maintain a degree of phenotypic plasticity in leaf morphology to help avoid or mitigate the effects of water-stressed environments. These variations include altered leaf shapes, trichome production, and waxy leaf cuticle development (Taiz et al., 2015). Stress response pathways can reduce leaf cell division and expansion, resulting in leaves with decreased surface area (e.g., from entire margins to lobed margins). Similar pathways may trigger increased production of trichomes, epidermal hairs that serve several functions including reducing leaf surface

temperatures, reflecting light radiation, and limiting water loss. A waxy cuticle helps combat water stress in two ways: waxier cuticles are shiny, and can reflect light radiation, and the multilayered waxes and extra-cellular hydrocarbons help reduce water loss from transpiration (Taiz et al., 2015). Initially, plasticity in leaf morphology might allow populations to survive periods of water stress in urban environments. If these conditions persist over generations, populations may be dominated by selectively advantageous phenotypes (Molina-Montenegro et al., 2011).

3.5.1 Water Stress Trials: Mortality and Fecundity

We expected urban plants to demonstrate higher survival rate and relative fitness (e.g., longer reproductive duration and increased seed pod number) compared to rural plants in all water stress treatments. However, there was no plant mortality in any trial, and very few plants produced flowers or seed pods. Plants that did reach the reproductive phase show no clear pattern across trials (Figure B.1). Almost all plants that flowered in the first trial were urban, whereas almost no urban plants flowered in the second trial. Furthermore, no plants from either category flowered in the drought treatment of the second trial, whereas very few did in the other trials. Some of the plants grown for the lab trials had the same seed origin (e.g., same seed collection population, same parent plant) as plants used during the reciprocal transplant experiments. It is notable that these populations/parent plants produced very few to no flowers during the lab trials, but had prolific flowering and seed pod production during the reciprocal transplant experiments. This indicates that some other factor (e.g. temporal or environmental) explains the low flowering rates observed in the lab for at least for those plants.

One possibility is that the lab trials were terminated before plants switched to the reproductive phase, as trials one and three occurred for 6 and 7 weeks, respectively. However, trial two occurred for 15 weeks, two weeks longer than the reciprocal transplant experiment, and the seedlings were similar in age at the onset. Therefore, an environmental factor is more likely responsible for low flower production. We subjected the plants to water stress, but perhaps the controlled conditions in the vegetation room were a more favorable (i.e., less-stressful) environment compared to conditions that occur in field sites. During the reciprocal transplant experiment, we saw a significant delay in flowering time when urban plants were grown in rural environments. There, urban plants invested more time in the vegetative growth phase, suggesting a release from stress. Perhaps both plant types experienced a similar scenario in the laboratory trials, and conditions were even less-stressful than rural field sites. Lack of mortality during the water stress trials also suggests that the treatments were not stressful enough. Since we were not able to statistically analyze and compare relative fitness of plants using flower and seed pod production, we used final plant size as a proxy for fitness (Younginger et al., 2017).

3.5.2 Water Stress Trials: Final Plant Size

We expected urban plants to be more tolerant to water stress, and that they would develop larger body-size despite stressful conditions via strategies of desiccation tolerance and avoidance. Conversely, we expected rural plants to be more susceptible to the physiological responses of drought evasion, resulting in smaller body sizes due to the decreased photosynthetic rate that occurs during stomatal closure.

The first trial showed a significant interaction between treatment and plant type. Urban final plant size was unaffected by all water stress treatments, and rural final plant

size was unaffected by flood and cyclic drought and flood. However, rural plant size was strongly impacted by drought conditions. This supports our hypothesis that urban plants are more tolerant to water stress conditions, particularly drought (Figure 3.3). Urban plants grew less in general, even in control treatments, suggesting that there may be a fitness cost of being able to resist drought stress. The second (Figure 3.4) and third trials (Figure 3.5) were inconclusive. Considering the results of the three trials together, it appears that both plant types are relatively unaffected by water stress.

3.5.3 Leaf Dry Matter Content and Specific Leaf Area

We expected that urban plants would show leaf traits better equipped to tolerate and avoid desiccation, and that urban plants would have higher LDMC and lower SLA values. Though plants grown in drought conditions did have leaves with greater LDMC compared to control plants, there was no difference in urban and rural leaves (Figure 3.6). SLA was similar for all plants (Figure 3.7).

3.5.4 Stomatal Density

Another leaf trait that helps plants compensate for water stress is stomatal density, as plants with fewer stomata would experience less water loss prior to stomatal closure. Indeed, we found that urban plants have reduced stomatal density under drought conditions whereas rural plants had increased densities (Figure 3.8). This is interesting, especially since both urban and rural final plant sizes were not significantly different under drought conditions in the third trial where leaf traits were measured. This difference in stomatal density might indicate that urban plants are able to rapidly increase photosynthetic rate once the drought stress is removed, but further testing is needed to confirm whether photosynthetic rates

actually vary. Another possibility is that urban plants have larger stomatal size compared to rural plants, which we did not measure.

3.5.5 Stellate and Single-haired Trichome Density

Trichomes serve several functions, including lowering leaf surface temperature and preventing water loss (Taiz et al., 2015). If urban plants are adapted to water stressed environments, we would expect higher trichome densities per leaf area. However, we found that urban and rural control plants have equivalent trichomes, and both respond similarly to drought conditions: both plant types produced fewer single-haired trichomes and more stellate trichomes under drought conditions (Figures 3.9-3.10). This may indicate that stellate trichomes play a role in regulating leaf temperature and preventing water loss. The stellate trichomes can be relatively large and have 3-7 branches that grow parallel to the leaf surface (Figure 3.14). Single-haired trichomes may serve a different primary function, such as herbivory defense (Cardoso, 2008), which could explain why their production decreases during drought stress.

3.5.6 Leaf Traits and Plant Size

Some leaf traits were strongly correlated with each other. We found that LDMC and SLA largely predicts final plant size of plants grown in drought conditions. Plants grown in drought had larger final plant sizes when leaves had higher LDMC and lower SLA.

3.5.7 Remarks

In the present study, we hypothesized that urban populations have undergone adaptive evolution in response to urban water stress regimes, and tested this in a series of laboratory

water stress trials. Overall, both rural and urban plants appear unaffected by water stress, and the species demonstrates plasticity in leaf traits in response to drought. Future directions include trials that subject plants to additional environmental variables to compare the relative fitness of urban and rural populations and leaf trait plastic responses.

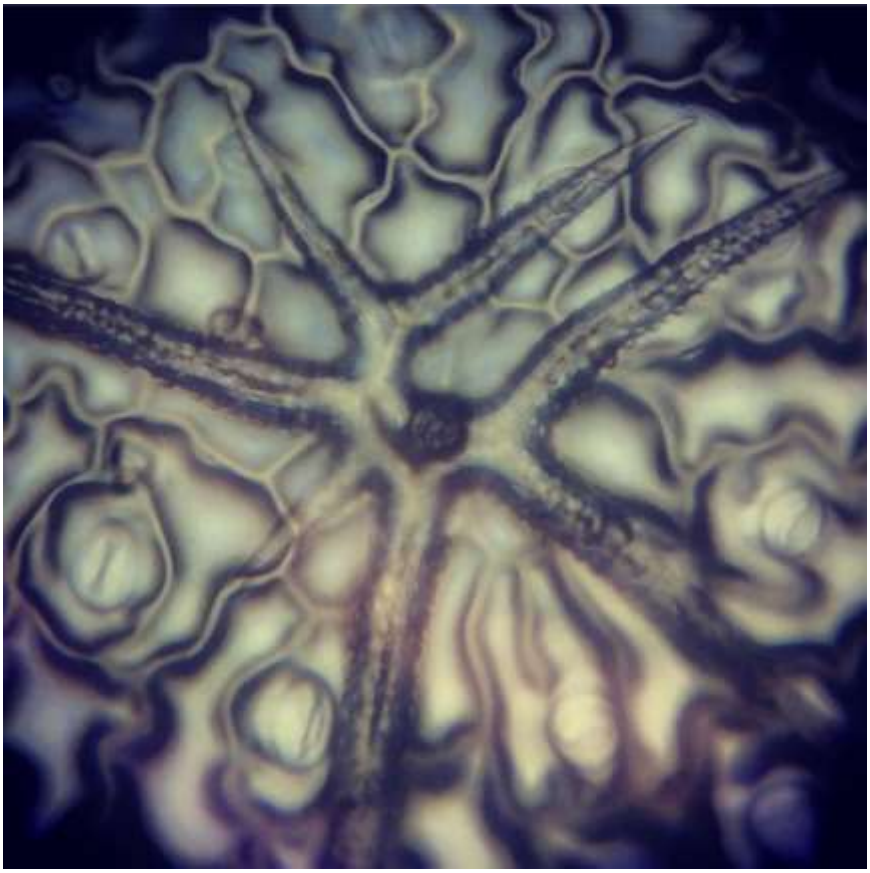

Figure 3.14 Example of stellate trichome with 7 branches (40X). They can be relatively large, having 3-7 branches that grow parallel to the leaf surface. Photo by Rebecca Panko.

CHAPTER 4

EFFECTS OF SALT STRESS ON PLANT GROWTH OF *CAPSELLA BURSA-PASTORIS* INDICATES SPECIES SALT-SENSITIVITY

4.1 Overview

Plants in urban environments must contend with soils that are often of poor quality, compacted, and polluted. Seasonal application of de-icing salts is a major contributing factor to urban soil pollution. The most frequently used de-icing salt is sodium chloride (NaCl), due to its relatively inexpensive cost. Saline soils exert physiological changes on plants that directly and indirectly affects their growth. *Capsella bursa-pastoris* is a cosmopolitan weed that regularly occupies roadsides and sidewalk cracks, and is very common in urban environments with high proportion impervious surfaces. Salt application is correlated with degree of urbanization, such that soils within cities are often more saline than in nearby suburban and rural locales.

In a field study (Chapter 2), we found evidence that populations of *C. bursa-pastoris* are locally adapting to conditions in urban environments. We tested these populations for differential tolerance to water stress using laboratory experiments (Chapter 3), and found that the species is relatively tolerant to water stress and demonstrates leaf trait plasticity in response to drought. Here, we hypothesize that populations are being shaped by urban soil salt stress. If urban populations are locally adapted to soil salt stress, we expect urban plants to 1) have higher relative fitness, 2) higher salt tolerance, and 3) exhibit leaf traits that better mitigate the effects of salt stress compared to rural plants when grown under increasingly saline conditions. We performed a laboratory trial with plants (n = 288) representing urban and rural populations from the New York metropolitan area.

Plants were grown under different salt treatments (0, 20, 40, 50, 60, 100, and 150 mM NaCl) for 7 weeks.

Both urban and rural plants had similar mortality rates: little to no mortality at low salt concentrations, and high mortality at high salt concentrations. Both plant types had decreased probability of survival as salt stress increased, with rural plants surviving slightly more. Urban and rural plants that survived the treatments had similar plant sizes within each treatment, and significantly larger plant sizes when grown under medium-high salt concentrations compared to controls. Plants displayed plasticity in leaf trait response to salt stress: as salinity increased, plants exhibited decreased leaf dry matter content, stomatal density, and stellate-trichome density, and increased specific leaf area. Single-haired trichome density increased in urban plants as salinity increased, whereas it decreased for rural plants. In most cases, leaf trait responses to salt stress contrasted the responses observed in plants grown in drought conditions.

Our results indicate that the species is 1) relatively salt-sensitive, despite its occurrence in habitats prone to de-icing salt pollution, and 2) highly plastic in leaf trait responses to abiotic stress, demonstrating differential responses depending on the source of stress.

4.2 Background

Plants growing in urban environments contend with a lot of stress, particularly if they grow along roadsides, within street tree pits, and through sidewalk cracks. These habitats are especially prone to factors such as soil compaction, vandalism, poor soil quality, and soil pollution (Cekstere & Osvalde, 2013; Kargar, Jutras, Clark, Hendershot, & Prasher, 2015). One main pollutant of urban soils is de-icing (or anti-icing) salt, which is liberally applied

both during and preceding winter storms to make paved surfaces safer for pedestrian and vehicular traffic (Czerniawska-Kusza, Kusza, & Dużyński, 2004). The degree of urban soil salinification increases with proportion impervious surface, such that denser cities experience higher levels of soil salt concentration compared to sprawling cities, suburban, and rural areas (Cunningham, Snyder, Yonkin, Ross, & Elsen, 2008).

There are several negative consequences to these large salt inputs into the urban environment, and their impacts occur at different spatial scales. Salt from urban areas can leach into waterways, turning freshwater sources non-potable and compromising the health of freshwater ecosystems well-beyond the city limits. Although salt application is seasonal, the ramifications to drinking water sources and freshwater organisms continues throughout the year (Kaushal et al., 2005). Within cities, salt pollution has an immediate impact on organisms that live in roadside and curbside habitats, especially plant species. It is well-established that soil pollution from de-icing salts can alter species compositions, impact plant growth and reproduction, and increase mortality of species living in close proximity to impervious surfaces (Cekstere, Nikodemus, & Osvalde, 2008; Czerniawska-Kusza et al., 2004; Eom, Setter, DiTommaso, & Weston, 2007; Li, Liang, Zhou, & Sun, 2014; Marosz & Nowak, 2008; Mastalerczuk, Borawska-Jarmulowicz, & Kalaji, 2019; Skultety & Matthews, 2017).

The most commonly applied de-icing salt is sodium chloride (NaCl), which comprises 98% of applications (Mastalerczuk et al., 2019). Sodium chloride is the least expensive salt for such purposes, hence its disproportionate use compared to other salts like calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) (New York State Department of Transportation, 2012). Given its dense nature and high proportion of

impervious surfaces, New York City has one of the highest volumes of salt application in the world, with the region using close to 1.5 million tons of salt in 2016-2019 across all five boroughs (Figure 4.1) (NYC OpenData, 2020).

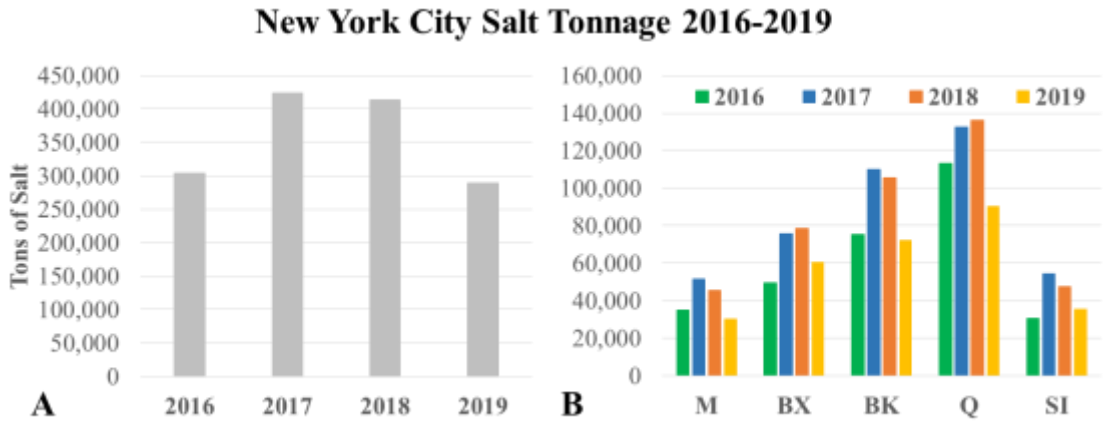

Figure 4.1 De-icing salt tonnage used in New York City from 2016-2019. (A) Tons of salt applied city-wide and (B) by borough. M = Manhattan, BX = Bronx, BK = Brooklyn, Q = Queens, SI = Staten Island. Data is from NYC Open Data, DSNY Salt Usage (2020).

Saline soils are those with an excess of mineral ions that can inhibit plant growth (Taiz et al., 2015). Salt that enters urban soils changes the physiological properties of the substrate, including the displacement of nutrient cations (e.g., K^{+}, Mg^{+2}, Ca^{+2}), reduction of soil permeability, and displacement and mobilization of metal ions, including heavy metals (Cunningham et al., 2008; Czerniawska-Kusza et al., 2004). Salt cations can also displace hydrogen cations, resulting in higher soil pH in urban soils (Kargar et al., 2015). The effects of salt pollution can be drastic, leading to severe injury and dieback of existing plants (Li et al., 2014) and driving species-assemblages of non-native, salt-tolerant plant communities along roadsides (Skultety & Matthews, 2017).

Plants require nutrients (e.g., N, P, K, Ca, and Mg) that are taken up as ions from the soil by the roots. These include many essential macro and micronutrients, and even low

concentrations of Na^+ and Cl^- are essential in certain physiological processes (Cekstere et al., 2008). However, when salt or heavy metal ion concentration reaches a species-dependent threshold, plants experience both nonspecific osmotic stress and specific ion effects. Ions bind to water molecules in the soil, and plant cells are not able to overcome the osmotic imbalance. This osmotic stress leads to water deficits and plants experience water stress similarly as if exposed to drought conditions, also known as "physiological drought" (Cekstere et al., 2008). Plants under osmotic stress quickly respond by closing stomata, limiting transpiration and photosynthesis, and therefore experience decreased shoot growth, leaf expansion, and lateral bud formation (Hooks & Niu, 2019). As toxic ions (e.g., Na^+ and Cl^-) accumulate in the substrate, they prevent uptake of essential nutrient ions. Plants grown in urban saline soils have exhibited tissues with increased Na^+ and Cl^- and decreased K^+, Mg^{+2}, Ca^{+2} ions (Cunningham et al., 2008). When toxic ions enter plant cells, it disrupts cytosolic activities and leads to nutrient deficiencies and cytotoxicity (Taiz et al., 2015). If ions reach high concentrations in the leaves, enzyme activity and chlorophyll concentration decreases, which interferes with photosynthesis and other biosynthetic pathways (Cekstere et al., 2008). Ultimately, leaves experience chlorosis, necrosis, and defoliation (Hooks & Niu, 2019).

Depending on species, plants have different relative tolerance to salt stress. Most plants cannot tolerate even low concentrations of salt (i.e., salt-sensitive plants), while others survive (i.e., salt-tolerant plants), and some even flourish (i.e., halophytes). There are several complex biosynthetic pathways that allow salt-tolerant plants and halophytes to combat the negative effects of saline soils. Some species prevent ions from entering at the roots, limiting the potential for cytotoxicity in other plant organs. Other species have roots

that allow ion entry, but avoid cytosolic ion accumulation via modifications such as salt glands that excrete salt from leaves and cells that sequester ions in vacuoles (Taiz et al., 2015). Similar mechanisms occur to prevent toxicity from heavy metals. Plants that can tolerate substrates with high levels of toxic ions can take advantage of roadside habitats prone to salt and heavy metal pollution, and ultimately may replace salt-sensitive species in these environments (Dudley, Jacobi, & Brown, 2014).

Several studies have examined herbaceous plant performance along roadways and in laboratory salt trials. A study comparing six herbaceous perennials found that species demonstrated a gradient of stress tolerance, and that the most tolerant species had much lower leaf Na^+ content compared to sensitive species (Eom et al., 2007), suggesting that those species either prevented ion intake at the roots or excreted ions at the leaf. A similar study using four herbaceous perennials found that all plants had decreased growth and biomass, and increased accumulation of Na^+ and Cl^- with higher salinity concentrations (Hooks & Niu, 2019). A common urban plant (*Trifolium repens* variety 'Daile') also accumulated Na^+ and Cl^- as substrate salinity level increased, and demonstrated a significant decline in biomass for all salt treatments (Cekstere, Karlsons, & Grauda, 2015). Sometimes phenology plays a role in salt tolerance, and plants are susceptible to salt damage at some stages but not in others. For example, Kentucky bluegrass (*Poa pratensis*) seeds did not germinate even in low concentrations (50mM) of NaCl, but mature plants produced equivalent shoots compared to controls at much higher concentrations (150 mM and 300 mM) (Mastalerczuk et al., 2019). Obviously, salt-tolerance or sensitivity is highly-species dependent. However, in habitats prone to salt pollution, it is logical to expect a high proportion of species to be salt-tolerant or halophytic over time. Indeed, these types of

plants have a positive relationship with heavily used roads in urban areas (Skultety & Matthews, 2017).

Capsella bursa-pastoris (L.) Medik. (Brassicaceae) (shepherd's purse) is a cosmopolitan weed that is often found in disturbed habitats like roadsides and sidewalk cracks. A ruderal species, it is short-statured (height 0.7 m) and is very common in urban environments where disturbance is frequent. In a previous reciprocal transplant study (Chapter 2), we found evidence that populations of *C. bursa-pastoris* are locally adapting to urban conditions in New York City. Since the species often grows in soils potentially influenced by de-icers, perhaps local adaptation is the result of long-term salt exposure in urban environments?

The species is not considered a halophyte (Orsini et al., 2010), yet there is evidence that it is capable of living in soils with high, toxic ion concentrations. Urban populations in the United Kingdom had increased leaf concentrations of Pb, Cd, Zn, and Cu compared to suburban and rural sites (Aksoy et al., 1999). During the reciprocal transplant study in New York City, the species was often observed co-occurring with *Lepidium latifolium* (Brassicaceae) (broadleaved pepperweed), which is known to be salt-tolerant (Dudley et al., 2014). *Capsella bursa-pastoris* is also a cold-season annual, such that it germinates and undergoes vegetative growth during the peak months for de-icing salt application.

We hypothesize that urban populations of *C. bursa-pastoris* have undergone adaptive evolution in response to urban salt stress. To quantify the effect of salt stress, we performed a laboratory trial with plants from urban and rural populations grown under different salt (NaCl) treatments: control (0 mM), low salt (20 and 40mM), medium salt (50 and 60 mM), and high salt (100 and 150 mM) concentrations. If urban populations are

adapted to salt stress, we expect that urban plants will 1) have higher relative fitness (e.g., reproductive duration and seed pod number), 2) exhibit higher salt tolerance (e.g., grow larger, have healthier leaves, live longer), and 3) have leaves that better mitigate the effects of osmotic stress (e.g., lower SLA, higher LDMC, fewer stomata, and more trichomes) compared to rural plants in all salt treatments.

4.3 Materials and Methods

4.3.1 Seed Collection

We collected seed pods from *Capsella bursa-pastoris* populations along an urban-rural gradient in the New York metropolitan area between May-June 2017. We used a stratified approach to find populations: we selected random points along the gradient within 60-km from Times Square (40°75'N, 73°98'W), followed roads that radiated out from each point, and collected seed pods from the first population located. We collected seed pods from each population (n = 24) from visibly healthy plants (n = 15; hereafter, "parent plants"). We removed seed pod infructescences from the base of each plant using scissors, and placed the cuttings into paper envelopes labeled with population number, GPS coordinates, and parent plant voucher number. Envelopes were stored in a dry, ventilated specimen room at the New Jersey Institute of Technology. We used QGIS (Version 2.18.3) (QGIS Development Team 2017) and 30 m resolution impervious surface land cover data from the 2011 National Land Cover Database (Multi-Resolution Land Characteristics Consortium www.mrlc.gov/data accessed on 21 February 2017) to analyze the proportion impervious surface (IS) of each population at a local scale (0.0081 km^2). We designated each population as urban (IS_{local} > 70%) or rural (IS_{local} < 50%).

4.3.2 Seedling Rearing

We sowed seeds (September 13-27, 2019) sequentially to acquire seedlings for 9 treatment blocks, such that all seeds for a given block were sown on the same day. Seeds were sown into 4-pack seed-starting containers (806 Inserts, Grower's Solution, Cookeville, TN, USA) using a germination mix (LM-18 Germination Mix, Griffin Greenhouse Supplies Inc, Tewksbury, MA, USA). The containers were placed into standard (55 cm x 28 cm) plant trays labeled with the seed sowing date. Trays were kept on metal plant shelves (Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA) under plant lights (Sun Blaze 44, ACF Greenhouses, Buffalo Junction, VA, USA) (16 h photoperiod). The seedlings were kept under germination hoods and trays were rotated twice a week.

Seedlings were reared under hoods for 2-weeks and then repotted into individual 4x4 plastic pots (04.00 SQ TRAD TW M POT, Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA). We filled each plastic pot with an equal volume of pre-moistened homogenized soil (Pro-Mix HP, Griffin Greenhouse Supplies Inc., Tewksbury, MA, USA). We carefully repotted each seedling, gently clearing loose dirt from the roots and dipping roots into a small water bath to remove as much propagation mix as possible without damaging the plant. We used a plastic fork to plant each seedling into the pot, using vouchered plastic labels to identify each plant (e.g., seed collection population ID, parent plant ID, seedling ID). We recorded the following information for each repotted seedling: date sown, date repotted, seedling voucher, seed collection population, parent plant number, number of leaves, longest leaf, chlorotic leaves, damaged leaves, dead leaves, and the lengths of the primary and secondary axes of the basal rosette. After repotting, the seedlings were acclimated under germination hoods for one week and without hoods for

two additional weeks. We monitored the temperature and humidity of the room (Humidity and Temperature Smart Home Environment System, AcuRite, Lake Geneva, WI, USA) and added humidifiers when needed. After the acclimation period, we began the salt stress trial.

4.3.3 Salt Stress Trial

The salt stress trial occurred from October 18 – December 6, 2019 and included 288 plants from 36 parent plants representing 11 urban populations and 10 rural populations. The trial involved different degrees of saline water treatments. Treatment blocks 1-6 included four salt-stress treatments: 50 mM NaCl, 100 mM NaCl, 150 mM NaCl, and a control using non-saline water. Due to high rates of mortality, treatment blocks 7-9 contained a less-severe group of treatments: 20 mM NaCl, 40 mM naCl, 60 mM NaCl, and a non-saline control. We used room temperature water for the salt trials, and pre-staged fresh water and pre-mixed solutions of salt water in opaque 5-gallon buckets with lids. All plants were bottom-watered during the trials, to prevent pouring treatment water directly onto leaves.

Every treatment block contained 4-trays, each representing a given treatment. Each tray contained 8 plants (e.g., 4 urban and 4 rural, representing 2 parent plants per plant type), such that a treatment block contained 32 plants. We recorded the following fitness data: alive or dead, longest leaf, number of leaves, number of dead leaves, number of chlorotic leaves, number of curled leaves, lengths of the primary and secondary axes of the basal rosette, number of flowers, number of seed pods, number of primary flower and secondary flower stalks, number of open seed pods, and number of stunted seed pods. Fitness measurements occurred on days 0, 7, 14, 21, 28, and 35 of the trial.

To begin a trial, we took plant fitness data (described above), rotated all plants within the tray, watered each plant to 200g, recorded pot weight, and poured 4 liters of pre-mixed treatment water into the bottom of the tray. After 1 h, we measured and recorded the electrical conductance (EC) of the tray water (EC-3 Water Quality Tester Electrical Conductivity Meter, range 0-9999 μS, accuracy ± 2%, HM Digital, Inc., Culver City, CA, U.S.A.). Trays and plants within trays were rotated twice a week. Every seven days, we recorded fitness data, pot weight, added 4 liters of fresh water, and recorded the EC of tray water one hour after it was added. Each treatment block was subjected to the salt trial for 7 weeks. We quantified final plant size as basal area of living plants at the end of the trial. Final salt content for each pot was calculated for all dead and surviving plants when the trial ended (data not shown). The soil from each pot was allowed to dry completely. Any remaining plant tissue was removed, and soil was homogenized using a 2 mm sieve. We used the 1:5 ratio and mixed 0.25 cups of homogenized soil with 1.25 cups of tap water. The mixture was stirred for 30 seconds and set aside for 30 minutes. After that time, the suspended solution was gently swirled and the EC of the solution recorded.

4.3.4 Leaf Trait Measurements

We harvested leaves from living plants at the end of the trial (t = 7 weeks) to conduct leaf trait analyses. We watered plants to 200g the day before leaf harvest to establish turgor. Leaves were harvested from plants (n = 86) that had at least three healthy (non-chlorotic) leaves. We removed leaves by cutting the petiole off from the base. We calculated leaf dry matter content and specific leaf area using the longest leaf, stomatal density using the second longest leaf, and trichome density and morphology using the third leaf (Cornelissen et al., 2003).

4.3.5 Leaf Dry Matter Content and Specific Leaf Area

Each leaf (n = 86) was weighed (PB4002-S Classic Plus scale, Mettler Toledo, Columbus, OH) immediately after it was removed from a plant. After weighing, we photographed the leaf (iPhone 5s, Apple Computer, Inc., Cupertino, CA) inside of a 10 cm x 15 cm frame labeled with the seedling voucher number. We put the leaf into a small paper envelope, labeled the envelope with the seedling voucher number, and placed the envelope into an 60ºC oven to dry for at least 72 h or until constant mass was achieved. We calculated leaf dry matter content (LDMC, mg g^{-1}) as:

$$\text{LDMC} = (\text{oven-dry mass}) / (\text{fresh mass}) \quad (4.1)$$

We used image analysis (ImageJ 1.47) (Schneider et al., 2012) to calculate leaf area. We set the measurement scale by drawing a line on the frame to a specific length (10 cm). The image was converted to grayscale (Image > Type > 8-bit) and made binary (Process > Binary > Make Binary). The area of the leaf was analyzed using the "*analyze particles*" command. We calculated specific leaf area (SLA, mm^2 mg^{-1}) as:

$$\text{SLA} = (\text{leaf area}) / (\text{oven-dry mass}) \quad (4.2)$$

4.3.6 Stomatal Density

We painted the lower epidermal surface of each leaf (n = 86) with clear nail polish immediately after it was removed from the plant. The leaves were allowed to air dry for at least 72 h. We removed the epidermal peels using clear transparent tape, and mounted the tape onto microscope slides labeled with the seedling voucher number (adapted from

Franks et al., 2009). Each microscope slide was viewed at 40X using a compound microscope (Nikon Alphaphot-2 YS2-H, Nikon Inc., Minato City, Tokyo, Japan) (example slide shown in Figure C.1). All stomata in frame were counted at three stratified points on the leaf (e.g., lower leaf, middle leaf, upper leaf) to obtain average stomata density per leaf. When stomata were cut off in field of view, we counted those on the bottom and right and disregarded those on top and left of the field of view.

4.3.7 Stellate and Single-haired Trichome Density

We used the upper epidermal surface of each leaf (n = 86) to quantify the density of stellate and single-haired trichomes (Figure 4.2) using a digital dissecting microscope (Nikon SMZ-25, Nikon Inc., Minato City, Tokyo, Japan) and associated software (NIS Elements 4.30, Nikon Inc., Minato City, Tokyo, Japan). We placed each leaf on the specimen stage and gently flattened it using a microscope slide. We drew a box (5.5 mm x 2 mm) at three stratified points on the leaf (e.g., lower leaf, middle leaf, upper leaf) and counted all single-haired and multi-branched (stellate) trichomes within the box to obtain average trichome density per leaf.

4.3.8 Statistical Analyses

Because our experimental design included random effects, we used linear and generalized linear mixed models analyses throughout, using the R package lme4 (Bates et al., 2014) in R (R software v.3.5.1, R Core Team, 2019). We first fitted a full model including plant type, salt treatment, and their interaction as fixed effects, and seed collection population, treatment block, and parent plant as random effects. We then reduced the full models by sequentially removing each random effect in turn. We then used Akaike's Information

Criterion (AIC; Akaike 1974) to choose the best fitting random effects model. We log-transformed response variables where necessary to meet the assumptions of normality and homoscedasticity, and confirmed the decision to log-transform by comparing AIC values.

To conduct hypothesis tests of fixed effects on continuous response variables, we performed F-tests using the Satterthwaite approximation for denominator degrees of freedom (Satterthwaite, 1946) with the R package lmerTest (Kuznetsova et al., 2017). To conduct hypothesis tests of fixed effects on logistic response variables, we used likelihood ratio tests (Buse, 1982), as methods for F-tests are not developed for this implementation of generalized linear mixed models.

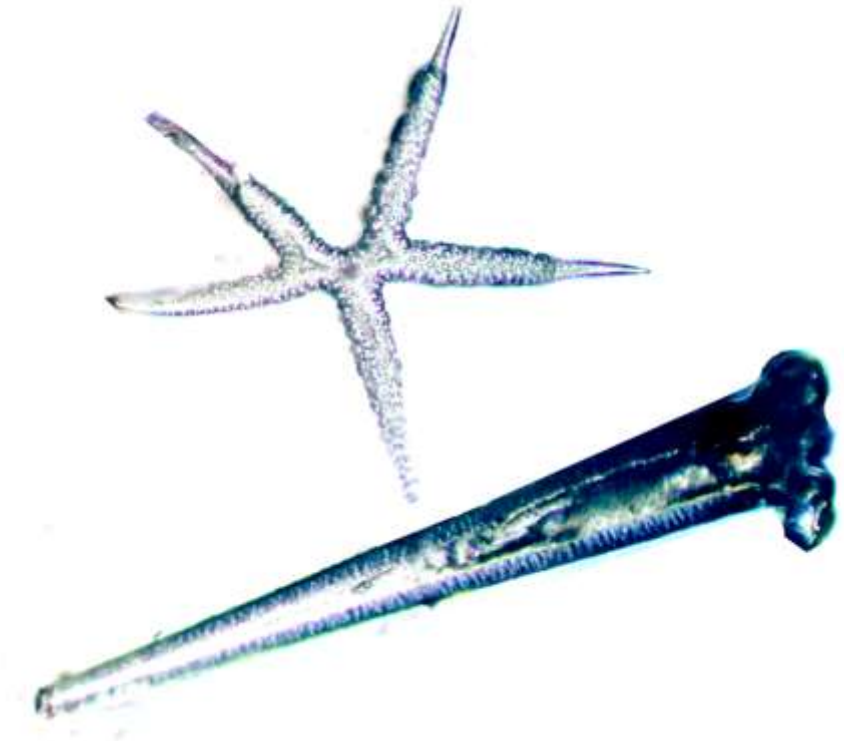

Figure 4.2 Example of stellate (top) and single-haired (bottom) trichomes found on *C. bursa-pastoris* (100X). Both trichomes are from the same leaf and adjacent to each other on the same microscope slide. Background has been removed from image and contrast increased to improve detail. Photo by Rebecca Panko.

We conducted orthogonal contrasts (Littell et al., 1996; Sokal & Rohlf, 1995) using the R package emmeans (Lenth, 2019) with Satterthwaite denominator degrees of freedom to explicitly compare responses of each plant type to each treatment. Lastly, we extracted estimated marginal means using the R package emmeans (Lenth, 2019) for presentation in figures. Due to high mortality rate in some treatments, we treated salinity as a continuous variable when analyzing leaf traits.

4.4 Results

4.4.1 Salt Stress Trial: Mortality and Fecundity

As mentioned above, treatment blocks 1-6 included four salt-stress treatments: 50 mM NaCl, 100 mM NaCl, 150 mM NaCl, and a control using non-saline water. Due to high rates of mortality in the highest salt treatments in blocks 1-6, treatment blocks 7-9 contained a less-severe group of treatments: 20 mM NaCl, 40 mM naCl, 60 mM NaCl, and a non-saline control. In addition, many seedlings from one population (population 23) began flowering before the treatments began, and those plants (n = 24) are not included in this analysis. Therefore, the number of plants per treatment varied in the following way: controls (n = 66), 20 mM NaCl (n = 20), 40 mM NaCl (n = 20), 50 mM NaCl (n = 46), 60 mM NaCl (n = 20), 100 mM NaCl (n = 46), and 150 mM NaCl (46 plants).

Many plants died during the experiment (38.2% of all plants, 101/264), the majority of which were in the 50 mM, 100 mM and 150 mM treatments. Controls and salt treatments 20 and 40 mM each had one plant die, and three plants died in the 60 mM treatment. The mortality rates were: controls (1.5%, 1/66), 20 mM (5%, 1/20), and 40 mM (5%, 1/20), 50 mM (41.3%, 19/46), 60 mM (15%, 3/20), 100 mM (71.7%, 33/46), and 150 mM (93.5%, 43/46). Both rural and urban plants had similar mortality in the 150 mM treatment, and of

plants that died, 51.1% were urban (22/43) and 48.9% were rural (21/43). About twice as many urban plants (63.6%, 21/33) died compared to rural plants (36.4%, 12/33) in the 100 mM treatment. Slightly more urban plants (57.9%, 11/19) died in the 50 mM treatment compared to rural plants (42.1%, 8/19).

Because there was so little mortality in the control and lowest salt treatments, we grouped treatments into categories to improve model convergence to compare survival probability. These categories are as follows: low salt (treatments 0, 20, and 40 mM NaCl), medium salt (treatments 50 and 60 mM NaCl) and high salt (100 and 150 mM NaCl). Statistical analysis of survival probability indicated that the best fitting model included only the random effect for treatment block and fixed effects for plant type and treatment type. The interaction between plant type and salt stress treatment was removed from the final model ($\Delta AIC = +3.0$, $\chi^2 = 0.99$, $df = 2$, $p = .608$). Plant type was retained in the model ($\Delta AIC = -0.69$, $\chi^2 = 2.7$, $df = 1$, $p = .101$), as was salt treatment ($\Delta AIC = -104$, $\chi^2 = 118$, $df = 1$, $p < .0001$). The model indicates that both urban and rural plants have decreased probability of survival as salt stress increases, with rural plants having a slightly better chance of survival (Figure 4.3).

Very few plants produced flowers (3% across all trials, 8/264). All plants that flowered were urban, except for one rural plant in treatment 100 mM NaCl. Two urban plants flowered in the control and 40 mM NaCl treatments, and one urban plant flowered in 20, 60, and 100 mM NaCl treatments. Only four plants (all urban) produced pods, one per control, 20, 40, and 100 mM NaCl treatments. No plants flowered in treatments 50 and 150 mM NaCl, and no pods occurred in treatments 50, 60, and 150 mM NaCl.

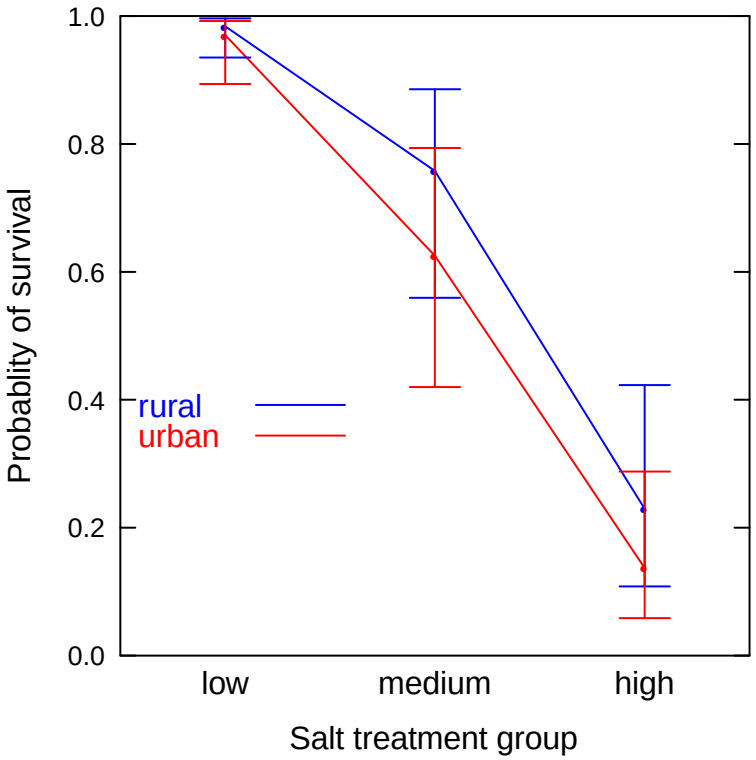

Figure 4.3 Effect of plant type (rural vs. urban) and salt treatment on survival probability. Model means and 95% confidence intervals shown. Because there was almost no mortality in the control and lowest salt treatments, we grouped treatments into categories to improve model convergence. These categories are as follows: low salt (treatments 0, 20, and 40 mM NaCl), medium salt (treatments 50 and 60 mM NaCl) and high salt (100 and 150 mM NaCl). The model indicates that both urban and rural plants have decreased probability of survival as salt stress increases, with rural plants having a slightly better chance of survival.

4.4.2 Salt Stress Trial: Final Plant Size

Since flower and seed pod production were low, we used final plant size as a proxy for fitness (Younginger et al., 2017). Statistical analysis of final plant size of surviving plants indicated that the best fitting model included the random effect of treatment block and parent plant. Type III F-tests showed a significant salt treatment effect ($p < .0001$, Table 4.1) (Figure 4.4). Plants that survived the treatments had similar plant sizes within each treatment.

Compared to controls, both plant types had significantly larger average plant size in the following treatments:

1) 50 mM NaCl (rural plants: 0.9 ± 0.187 cm^2; $t_{1,137}$ = 4.868, p <.0001 | urban plants: 0.9 ± 0.182 cm^2; $t_{1,133}$ = 5.021, p <.0001),

2) 100 mM NaCl (rural plants: 0.96 ± 0.202 cm^2; $t_{1,136}$ = 4.774, p <.0001 | urban plants: 1.3 ± 0.345 cm^2; $t_{1,140}$ = 3.785, p < .001),

3) 150 mM NaCl (rural plants: 1.38 ± 0.578 cm^2; $t_{1,135}$ = 2.388, p = .0183 | urban plants: 0.8696 ± 0.351 cm^2; $t_{1,140}$ = 2.479, p = 0.0144).

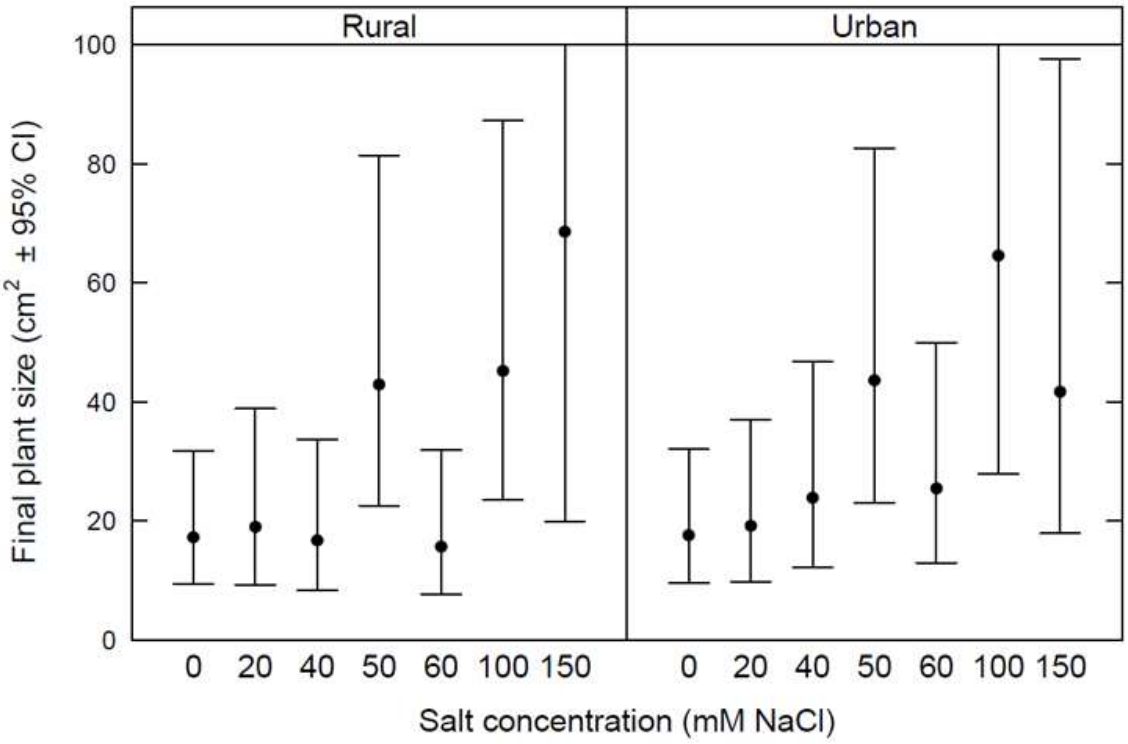

Figure 4.4 Effect of plant type (rural vs. urban) and salt treatment (0, 20, 40, 50, 60, 100, 150 mM NaCl) on final plant size of plants that survived treatments. Model means and 95% confidence intervals shown. Type III F-tests showed a significant salt treatment effect (p < 0.0001, Table 4.1). Raw data is shown in Figure C.2

Table 4.1 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Salt Stress Treatment and Plant Type on Final Plant Size

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Treatment	20.0105	3.3351	6	129.636	11.3669	<.0001
plant_type	0.1376	0.1376	1	46.241	0.469	0.4968
treatment:plant_type	1.3483	0.2247	6	126.133	0.7659	0.5981

Note: final model included treatment block and parent plant as random effects.

4.4.3 Leaf Dry Matter Content

Statistical analysis of LDMC indicated that the best fitting model included treatment block and seed collection population for random effects. Type III F-tests showed a significant treatment effect ($p < 0.0001$, Table 4.2). Both plant types show a negative correlation between LDMC and salinity concentration (Figure 4.5).

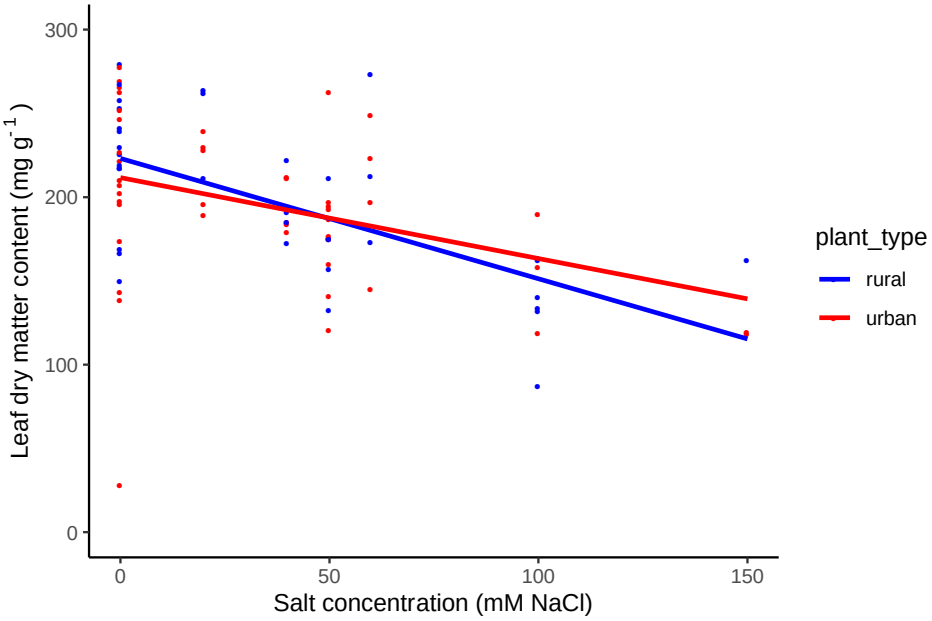

Figure 4.5 Effect of plant type (rural vs. urban) and treatment (0, 20, 40, 50, 60, 100, 150 mM NaCl) on LDMC (mg g^{-1}). Model predictions and raw data shown. Type III F-tests showed a significant treatment effect ($p < .0001$, Table 4.2). Both plant types had LDMC decrease as salinity concentration increased.

Table 4.2 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Salt Stress Treatment and Plant Type on Leaf Dry Matter Content

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatnum	41897	41897	1	68.065	29.3541	<.0001
plant_type	1094	1094	1	46.775	0.7663	0.3858
treatnum:plant_type	1648	1648	1	66.619	1.1546	0.2865

Note: final model included treatment block and seed collection population as random effects.

4.4.4 Specific Leaf Area

Statistical analysis of SLA indicated that the best fitting model included treatment block and seed collection population for random effects. Type III F-tests showed a significant treatment effect ($p < .001$, Table 4.3). Both plant types show a positive correlation between SLA and salinity concentration (Figure 4.6).

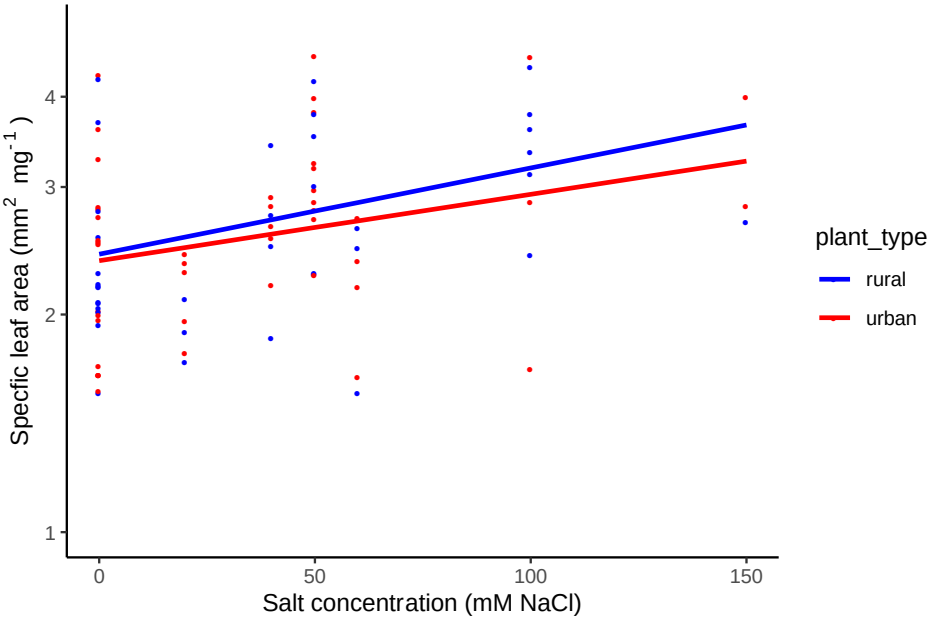

Figure 4.6 Effect of plant type (rural vs. urban) and treatment (0, 20, 40, 50, 60, 100, 150 mM NaCl) on SLA (mm^2 mg^{-1}). Model predictions and raw data shown. Type III F-tests showed a significant treatment effect ($p < .001$, Table 4.3). Both plant types show a positive correlation between SLA and salinity concentration.

Table 4.3 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Salt Stress Treatment and Plant Type on Specific Leaf Area

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatnum	0.66071	0.66071	1	62.691	14.7598	0.0003
plant_type	0.0027	0.0027	1	39.374	0.0604	0.8071
treatnum:plant_type	0.01143	0.01143	1	61.559	0.2553	0.6151

Note: final model included treatment block and seed collection population as random effects.

4.4.5 Stomatal Density

The best fitting model for stomatal density included treatment block and seed collection population for random effects. Type III F-tests showed a significant treatment effect ($p < .001$, Table 4.4). Both plant types show a negative correlation between stomatal density and salinity concentration (Figure 4.7).

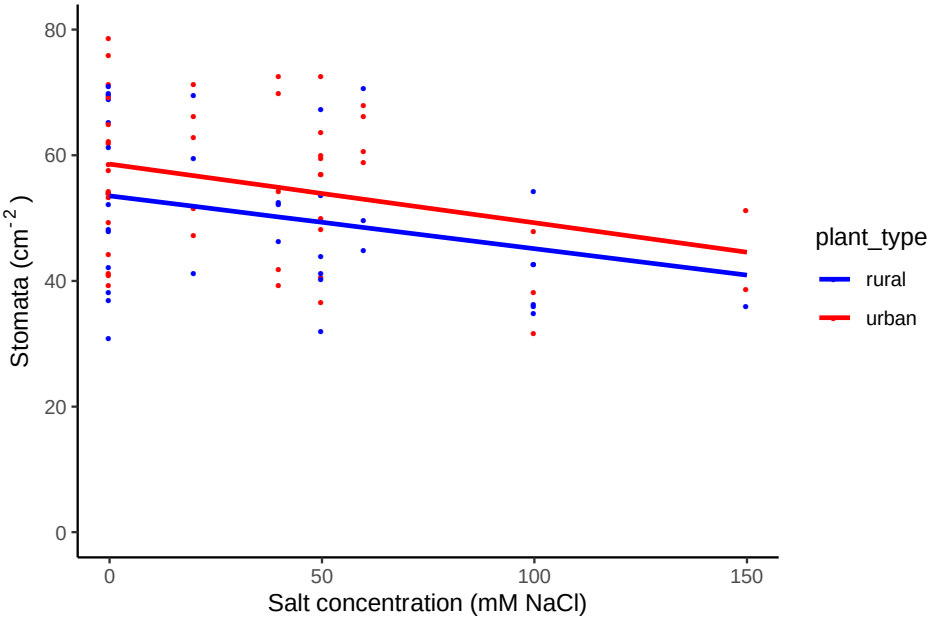

Figure 4.7 Effect of plant type (rural vs. urban) and treatment (0, 20, 40, 50, 60, 100, 150 mM NaCl) on stomatal density (cm^{-2}). Model predictions and raw data shown. Type III F-tests showed a significant treatment effect ($p < 0.001$, Table 4.4). Both plant types show a negative correlation between stomatal density and salinity concentration.

Table 4.4 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Salt Stress Treatment and Plant Type on Stomatal Density

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatnum	874.73	874.73	1	62.119	12.717	0.0007
plant_type	149.92	149.92	1	29.449	2.1796	0.1504
treatnum:plant_type	2.51	2.51	1	61.377	0.0365	0.8491

Note: final model included treatment block and seed collection population as random effects.

4.4.6 Stellate and Single-haired Trichome Density

The best fitting model for stellate trichomes included treatment block and seed collection population for random effects. Type III F-tests showed a significant treatment effect ($p < .0001$, Table 4.5). Both plant types had decreased stellate production as salinity increased, with rural plants tending to produce slightly less (Figure 4.8). The best fitting model for single-haired trichomes included treatment block and parent plant for random effects. Type III F-tests showed a significant interaction between treatment and plant type ($p < 0.05$, Table 4.6). Urban plants exhibited slightly more single-haired trichomes as salinity increased, whereas rural plants had decreased single-hair trichome production (Figure 4.9).

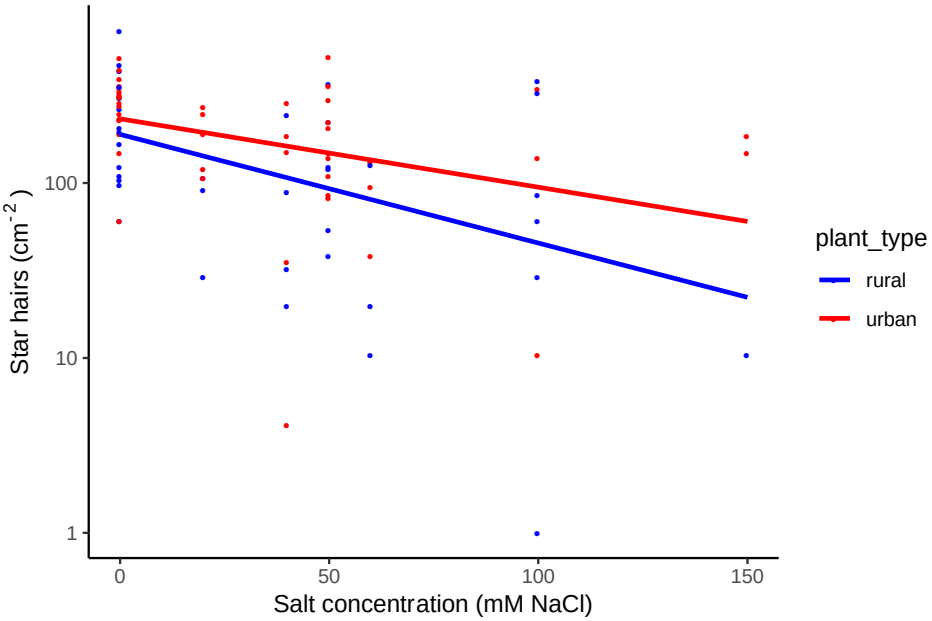

Figure 4.8 Effect of plant type (rural vs. urban) and treatment (0, 20, 40, 50, 60, 100, 150 mM NaCl) on stellate trichome production. Model predictions and raw data shown. Type III F-tests showed a significant treatment effect ($p < .0001$, Table 4.5). Both plant types had decreased stellate production as salinity increased, with rural plants tending to produce slightly less.

Table 4.5 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Salt Stress Treatment and Plant Type on Stellate Trichome Density

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatnum	15.406	15.406	1	63.521	26.1021	<.0001
plant_type	0.2009	0.2009	1	43.818	0.3404	0.5626
treatnum:plant_type	0.8076	0.8076	1	62.468	1.3683	0.2465

Note: final model included treatment block and seed collection population as random effects.

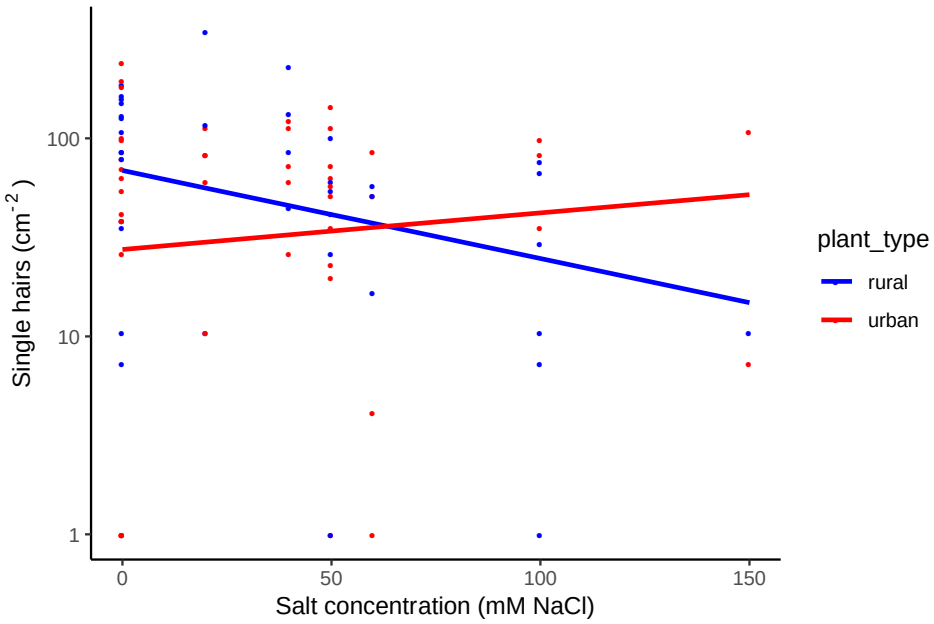

Figure 4.9 Effect of plant type (rural vs. urban) and treatment (0, 20, 40, 50, 60, 100, 150 mM NaCl) on single-haired trichomes. Model predictions and raw data shown. Type III F-tests showed a significant interaction between treatment and plant type ($p < .05$, Table 4.6). Urban plants exhibited slightly more single-haired trichomes as salinity increased, whereas rural plants show decreased single-hair trichome production.

Table 4.6 Type III Analysis of Variance Table with Satterthwaite's Method for Effects of Salt Stress Treatment and Plant Type on Single-haired Trichome Density

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
treatnum	0.9576	0.9576	1	57.892	1.0414	0.3117
plant_type	3.7166	3.7166	1	32.239	4.0419	0.0528
treatnum:plant_type	5.6371	5.6371	1	58.208	6.1306	0.0162

Note: final model included treatment block and parent plant as random effects.

4.5 Discussion

Urban environments have higher proportion impervious surfaces compared to suburban and rural locales, and thus urban soils are more often polluted by de-icing salts. This is particularly true for soils along roadsides and sidewalks, habitats where *Capsella bursa-pastoris* regularly occurs. Given the species' 1) abundance in roadside and sidewalk-crack habitats, 2) ability to sequester heavy metal ions out of urban soils (Aksoy et al., 1999), and 3) observed co-occurrence with known salt-tolerant species (e.g., *Lepidium latifolium*), we proposed saline soils as a selective driver of urban populations of *C. bursa-pastoris*.

4.5.1 Salt Stress Trial: Mortality and Fecundity

We expected urban plants to demonstrate higher survival rate compared to rural plants in all salt treatments. However, survival was similar for both plant types: almost no mortality at the lowest salt concentrations (0-40 mM), and almost complete mortality (93.5%) at the highest salt concentration (150 mM). Opposite to our expectations, nearly twice as many urban plants died compared to rural plants at the second highest concentration (100 mM), and our model indicates that rural plants tend to have slightly better survival rate as salinity increases (Figure 4.3). Since *Capsella bursa-pastoris* has been shown to have higher concentrations of foliar heavy metal ions in urban environments, perhaps these populations are also taking in higher concentrations of Na^+ and Cl^- ions. Indeed, some urban plant leaves appeared to be excreting salt through leaf glands (Figure 4.10). This could serve to relieve osmotic stress at the root, decreasing the effects of "physiological drought," but may ultimately accumulate to toxic levels in these plants. Conversely, perhaps rural populations have roots that better exclude salt ions.

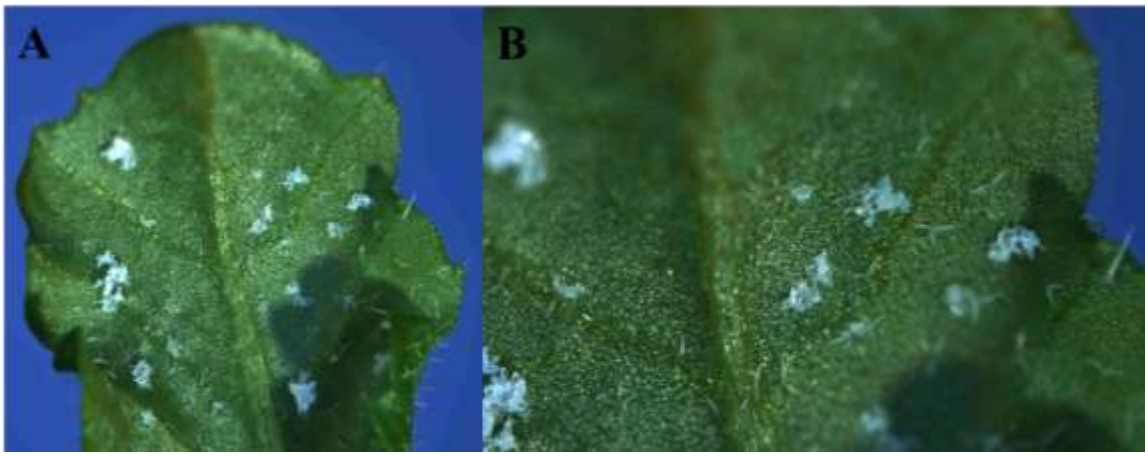

Figure 4.10 Example of salt on leaves from urban plants. Shown is plant voucher 2.33.4 at (A) 1.5X and (B) 3X. Photos by Rebecca Panko.

We also expected urban plants to demonstrate higher relative fitness (e.g., longer reproductive duration and seed pod number) compared to rural plants in all salt stress treatments. However, we could not statistically compare these traits due to low flower and seed pod production. Only 3% of plants produced flowers during the salt trial. The trial ended due to time constraints, and perhaps more plants would have produced flowers if given more time. However, low flower production also occurred during laboratory water stress trials (Chapter 3) that lasted 15 weeks, while many plants produced flowers and seed pods during the 13-week reciprocal transplant experiment (Chapter 2). Alternatively, low flower production during the salt stress trials may be due to an environmental factor. It is possible that the controlled conditions in the vegetation room were relatively less-stressful environments compared to conditions that occur in field sites. Mortality rate during the salt stress trials indicates that plants were adequately stressed at high salt concentrations. Of the few plants that flowered during the salt stress trial, it is interesting that the majority (7/8) were urban, that only urban plants produced pods, and that one even produced seed pods in a relatively high salt concentration (100 mM NaCl).

4.5.2 Salt Stress Trial: Final Plant Size

We used final plant size of surviving plants as a proxy for fitness, as larger plants are generally shown to have a greater reproductive output (Younginger et al., 2017). We expected urban plants to be more tolerant to salt stress, and therefore to develop larger body sizes in all salt treatments. However, surviving plants had similar sizes within treatments, and the only significant differences were treatment effects (Figure 4.4).

There have been many studies across plant taxa that examine how salt stress affects performance. Many studies report decreased biomass as soil salinities increase (Cekstere et al., 2015; Czerniawska-Kusza et al., 2004; Ju, Yeum, Son, & Yoon, 2017; Marosz & Nowak, 2008), although these studies mostly focus on tree species. We found the opposite response in our species, as average plant size is positively correlated with increased salt concentration for plants that survived. Control plants and those at low salt concentrations had relatively small (~20 cm^2) average final plant sizes, whereas average sizes at relatively high concentrations were almost doubled (>40 cm^2). However, as shown by the wide confidence intervals, this effect is not conclusive (Figure 4.4). Much of the variance is due to how many plants died at higher concentrations, such that few individuals were left to represent size (See Appendix C, Figure C.2). Nevertheless, those plants that did survive the high salt concentrations were larger than controls and plants at low salt concentrations. Perhaps these plants demonstrate a stronger plastic response to salt stress, which allows them not only to survive but also invest resources into vegetative growth. This could indicate that salt exposure over generations may result in selectively larger plants.

4.5.3 Leaf Trait Overview

We expected urban plants to have leaf traits that better mitigate the effects of osmotic stress compared to rural plants. However, surviving rural and urban plants had similar leaf trait responses as salinity concentration increased. These responses indicate that the species is highly plastic in leaf trait morphology during salt stress. We expected plants that survived salt treatments to demonstrate leaf morphologies that better mitigate the osmotic stress (e.g., "physiological drought") associated with salt stress (Cekstere et al., 2008), and expected those leaves to have: higher LDMC, lower SLA, fewer stomata, and more trichomes. Survivors instead had the following leaf traits as salinity increased: lower LDMC, higher SLA, fewer stomata, and fewer trichomes (Figure 4.5-4.9). These responses differ from plants grown in drought treatments (Chapter 3). In drought, leaf plastic responses resulted in higher LDMC. Perhaps lower LDMC (Figure 4.5) during salt stress indicates that leaves are sequestering water when confronted with salt stress. Alternatively, it could indicate cytotoxicity and destruction of plant cell organelles. Similarly, we observed no change in SLA in plants grown in drought, whereas SLA increased under salt stress (Figure 4.6). Stomatal density did decrease under salt stress (Figure 4.7), as expected, whereas only urban plants had a similar decrease in drought conditions. Lastly, we see an opposite effect on trichome production under salt stress compared to drought stress.

Plants produced fewer stellate trichomes in salt stress (Figure 4.8), and more under drought stress. Single-haired trichomes decreased for urban plants and increased for rural plants under salt stress (Figure 4.9), whereas both plant types produced more single-haired trichomes in drought treatments. These contrasting results suggest that plants are up-taking

salt ions, not preventing ion entry at the roots. Clearly *C. bursa-pastoris* is very plastic in stress response, and the leaf trait response varies depending on the source of stress.

4.5.4 Remarks

We hypothesized that urban populations of *Capsella bursa-pastoris* have undergone adaptive evolution in response to urban soil salt pollution, and tested this in a laboratory trial. Plant mortality was similar for both urban and rural plant types, with rural plants having slightly lower mortality with exposure to salt. There is some evidence that the species can tolerate the input of low amounts of soil salt, but demonstrated almost complete mortality (93.5%) at 150 mM NaCl. As a comparison, close relative *Arabidopsis thaliana* (which is considered salt sensitive) has been shown to experience 50% mortality at 150 mM NaCl (Orsini et al., 2010). Whereas another close relative, *Thellungiella halophila* (a known halophyte), can still reproduce at extremely high salinities (500 mM NaCl) (Inan et al., 2004). Despite being salt-sensitive, survivors of the salt treatments demonstrate that *Capsella bursa-pastoris* shows remarkable plasticity in leaf trait responses to salt stress. Individuals with the greatest plasticity might have the selective advantage in environments with high levels of abiotic stress.

4.5.5 Future Directions

We would like to analyze the final salt content data we collected from plants that lived and died during this trial. This future analysis could help us better understand results contained here. These data were not yet analyzed because of interference of lab work due to COVID 19.

CHAPTER 5

CONCLUSION

The goal of this work was to 1) quantify the relative fitness of urban and rural populations of shepherd's purse (*Capsella bursa-pastoris*) in the New York metropolitan area, 2) determine whether they are locally adapting to conditions along the urban-rural gradient, and 3) identify which environmental factors are responsible for driving the selection. We hypothesized that urban populations have undergone adaptive evolution in abiotic stress response via prolonged exposure to urban abiotic stress, namely water stress and soil salinity. We chose *C. bursa-pastoris* as a study species because it occurs in cities worldwide, self-pollinates, has short generation times, and is commonly found in disturbed sites (e.g., along roads, in sidewalk cracks) that are likely to experience flooding, drought, and de-icing salt soil pollution. We conducted three experiments to test our hypothesis.

In a reciprocal transplant experiment (Chapter 2), we found evidence for local adaptation, as urban populations produced more seed pods at home compared to away sites, and both plant types had longer reproductive durations at home sites. These results indicate that wild populations of shepherd's purse are locally adapted to urban conditions, but which abiotic stress variable (or variables) is driving this adaptation?

Based on the results of a common-garden laboratory trial, it isn't water stress (Chapter 3). No plants died in any water stress treatment, and very little flowering occurred. We used final plant size as a proxy for fitness, and there were no conclusive trends across three trials. Generally, though, plants appeared unaffected by water stress. Both plants showed plastic leaf trait responses under drought stress.

Both plant types also had similar responses to salt-stress within treatments (Chapter 4): plant size increased as salt-concentration increased, and mortality was high at both medium and high salt concentrations. Furthermore, this cosmopolitan weed, that often lives along roadsides and sidewalk cracks, is surprisingly salt-sensitive. Leaf trait responses of surviving plants in salt treatments were opposite to those observed in drought treatments, highlighting how plastic the species is in response to abiotic stress. Our results do not implicate either water or salt-stress in driving the adaptation we observed in urban populations during the field experiment. Therefore another environmental variable is responsible, or selection is occurring via a combination of several variables, the latter of which is more likely. Urban environments are heterogeneous and highly complex systems that present a multitude of stress factors on urban populations. Even though we exposed laboratory plants to a single source of stress during the trials, perhaps the controlled lab environment is simply relatively stress-free compared to life in the real world.

5.1 Species Morphological Variation

Capsella bursa-pastoris displays an incredible amount of plasticity, morphological diversity, and naturally exhibits a lot of variation (Iannetta et al., 2007; Neuffer et al., 2018; Shull, 1909). Perhaps individuals with the greatest degree of plasticity are the fittest, and have allowed the species to reach global distributions in disturbed habitats.

5.1.1 Botanical Curiosities

Several types of morphological variation is well-documented in *C. bursa-pastoris*, and we observed some additional curiosities during the course of this study. The first example is stomatal-twins (Figure 5.1), where two separate stomatal apparatuses physically touch

guard cells. This characteristic was observed on one leaf of the 136 leaves used to perform stomatal density counts. It originated from a rural plant grown under 100 mM NaCl, and the leaf had many stomatal twins. This type of "stomatal clustering" has been reported in at least 60 species over the last several decades (Dehnel, 1961; Gan et al., 2010) (Figure 5.2). The phenomenon is correlated with environmental stress, including water and drought stress (Gan et al., 2010). This occurs because many genes and transcription factors responsible for stomatal development are also involved in both abiotic and biotic stress response (Figure D.1). To the best of our knowledge, we are the first to report stomatal twins in *Capsella bursa-pastoris*.

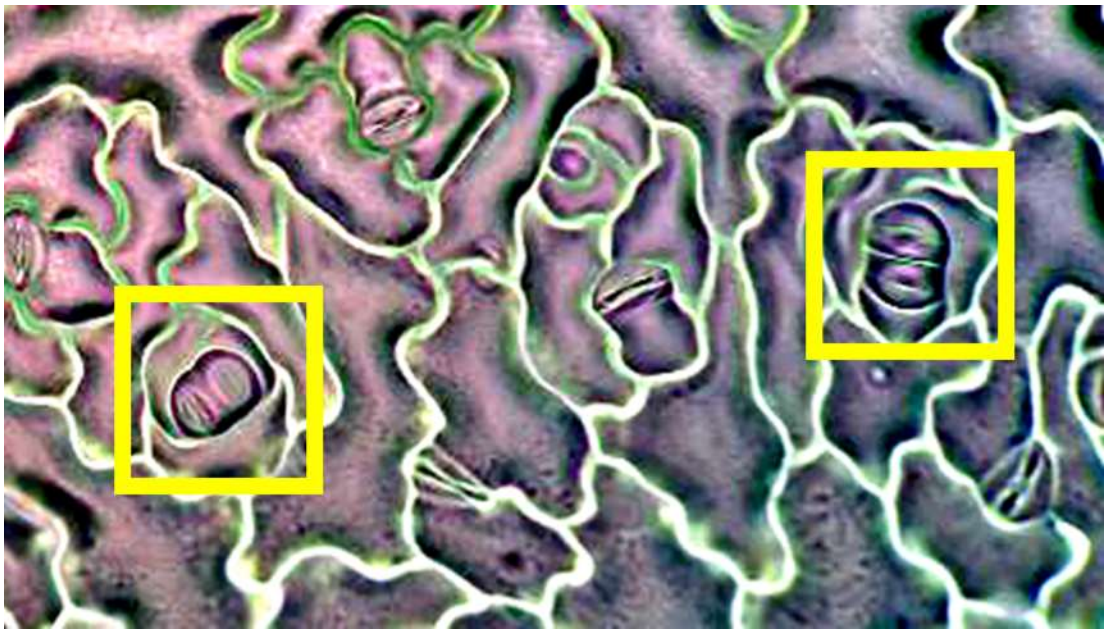

Figure 5.1 Example of stomatal twins (yellow boxes) seen on a leaf from a rural population (#6), plant parent 87, grown under 100 mM NaCl treatment (40X). This leaf had many clustered stomata. Contrast is increased to improve detail. Photo by Rebecca Panko.

Another curious variation we observed was the presence of hook-like and spine-like trichomes at the leaf margins of some plants (Figure 5.3). These likely serve in

discouraging herbivores, some of which may get trapped in trichome hooks (Cardoso, 2008; Yasuhiro, Shimizu-Inatsugi, Yamazaki, Shimizu, & Nagano, 2019). Hooked trichomes were only observed on leaves from rural plants grown in drought, whereas spine trichomes occurred on both plant types and treatments.

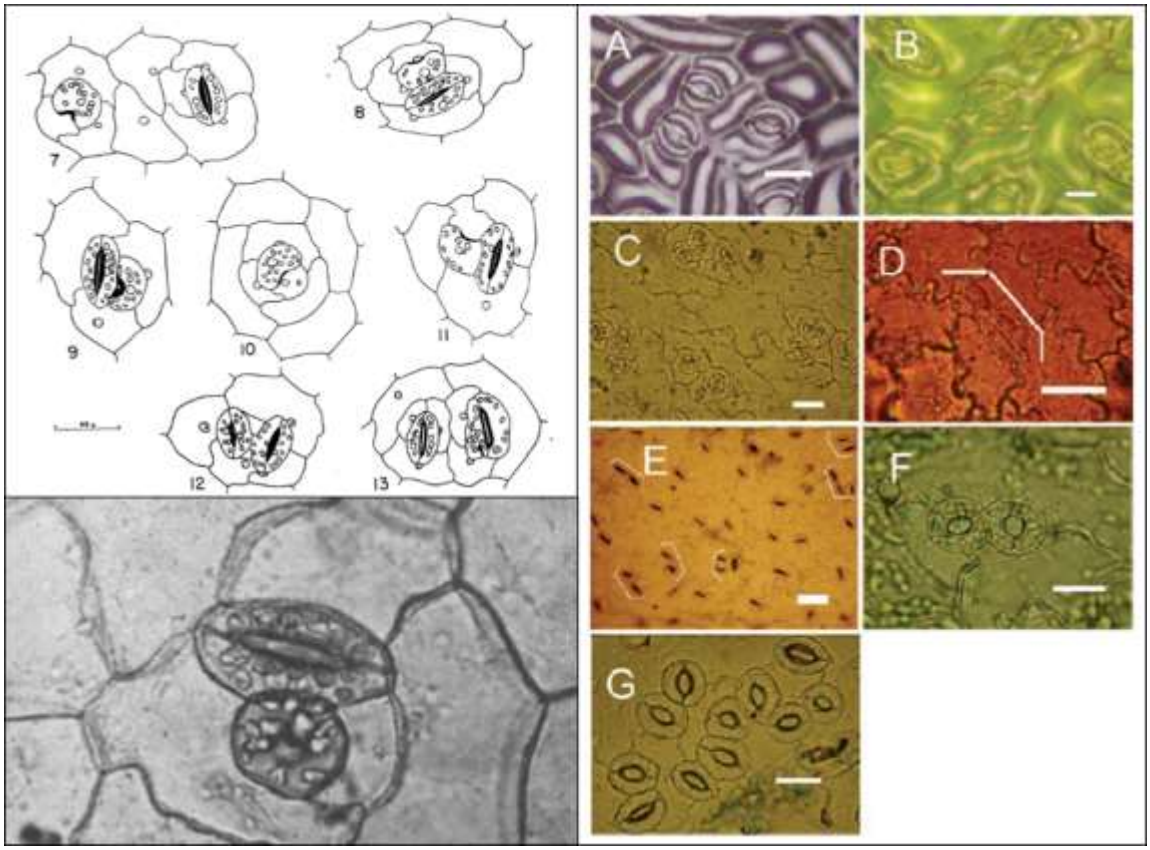

Figure 5.2 Stomatal clustering has been described in more than 60 species over the last 60 years: (left) sketches and photo of stomata in close proximity to one another in *Begonia aridicaulis* (Dehnel, 1961) and (right) examples of "stomatal clustering" in 7 plant species (Gan et al., 2010).

Lastly, we also observed variation in seed pods. *Capsella bursa-pastoris* has seed pods (i.e., fruits) called silicles: two carpels that split away (i.e., dehisce) from a central replum (Figure 5.4, A and B). We observed some plants bore seed pods with both increased

and decreased carpel numbers (Figure 5.4, C, D, F), and even some twin pods attached to the same peduncle (Figure 5.4, E).

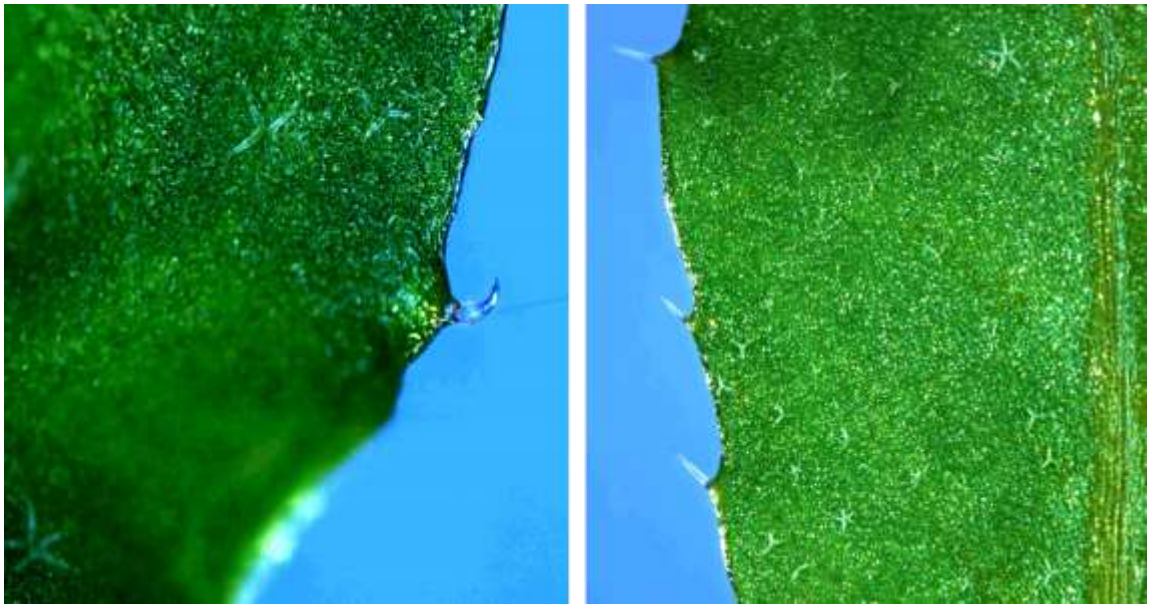

Figure 5.3 Examples of leaf margin trichomes. (left) hook-like trichome on rural leaf grown in drought (population 19, parent plant 281) (5X) and (right) spine-like trichome on urban leaf grown in control water treatment (population 21, parent plant 302) (2.5X).

5.2 Plant-Animal Interactions

Since *Capsella bursa-pastoris* self-pollinates without the need for biotic pollinators, some might disregard its role in plant-animal interactions within urban ecosystems. However, we observed two examples of such an interaction. Ladybugs (Coleoptera: Coccinellidae) were occasionally seen on the species (Figure 5.5) during the reciprocal transplant experiment. Aphids, which are common prey for ladybugs, were also (separately) observed. There was also one instance in which (what appeared to be) ladybug eggs were attached to a seed pod (Figure 5.5).

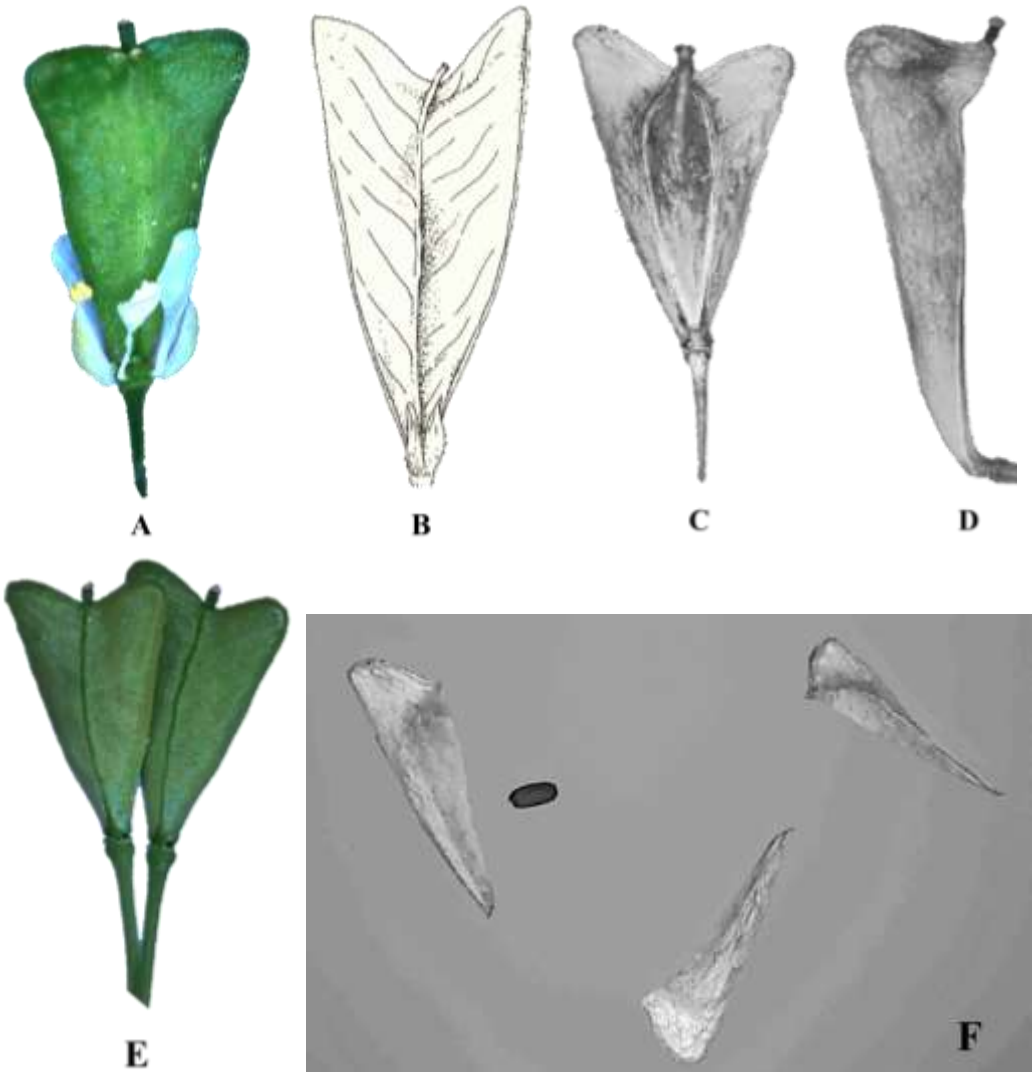

Figure 5.4 Examples of seed pod variation. (A) developing silicle with floral structures still attached at base, (B) mature silicle, (C) silicle with 3 carpels, (D) silicle with one carpel, (E) two silicles per peduncle, (F) dissected 3 carpel silicle and a seed. (Mature silicle drawing in B from Levine, 1995) Photographs A, C-F by Rebecca Panko.

The more common interaction the species likely experiences is herbivory, though we did not observe any mechanical damage from insects in the field. There was one instance where people were collecting large amounts of the plants within Lindower Park (Brooklyn, NY). All parts of shepherd's purse are edible to humans (though it causes contact dermatitis in some individuals) (Foster & Duke, 1990). However, there was one unexpected herbivore observed: *Columba livia domestica* (domestic pigeon). Pigeons were

seen systematically eating every seed pod off of plants located in the Lower East Side, Manhattan, NY (Figure 5.6). So there a possibility the species is actually bird-dispersed.

Figure 5.5 Example of an observed plant-animal interaction (left) ladybugs were occasionally seen on *C. bursa*-pastoris and (right) what appears to be ladybug eggs attached to a seed pod. Photos by Rebecca Panko.

Figure 5.6 Seed pod herbivory by *Columba livia domestica* (domestic pigeon): (left) *Capsella bursa-pastoris* plant bearing many seed pods before pigeon herbivory, (center) all seed pods removed via pigeon herbivory, (right) pigeon (circled) walking away after consuming seed pods off several plants. Photos by Rebecca Panko (18 May 2017).

5.3 Urban Plant Evolution: Suggested Next Steps

The plant kingdom is diverse and cities are heterogeneous. We need to conduct field studies, at different spatial and temporal scales, that examine selection in urban environments across plant taxa that differ in: 1) life history traits (e.g., annual, perennial, herbaceous, woody, self-pollinating, self-incompatible), 2) ecological strategies (e.g., stress-tolerant, stress-resistant), and 3) seed dispersal mechanisms and pollination syndromes (biotic and abiotic), to understand the macroevolution of plants in an increasingly urbanized world.

APPENDIX A

SITE INFORMATION

This is additional site information for the reciprocal transplant sites. The methods for heavy metal analysis are given in A.1, and results shown in Table A.4. Figure A.2 and A.3 show percent green cover and soil compaction, respectively.

A.1 Analytical Methods for Soil Heavy Metal Analyses

(Performed by Rodríguez-Freire Lab, NJIT)

Heavy-metal composition of all soil samples was measured using an Agilent 7500i Benchtop Inductively Coupled Plasma ionization (ICP) coupled with either a Mass Spectrometer (MS). Soil samples were air-dried for 48 h and crushed with a mortar and pestle for homogenization. One gram of the dried solids was weighed, the weight recorded, and added to the digestion tube, with 2 mL HNO3 (UHP) and 1 mL HCl (UHP). The mixture was digested in a Digi prep MS SCP Science block digester for 60 min at 65° C and then for an additional 60 min at 80° C. The digested sample was diluted with deionized water to 10 mL, and then filtered through a 0.45 µm filter (DigiFILTER 0.45 µm Hydrophilic Teflon®) to remove any particulate matter, and analyzed in the ICP-MS for trace elements constituents.

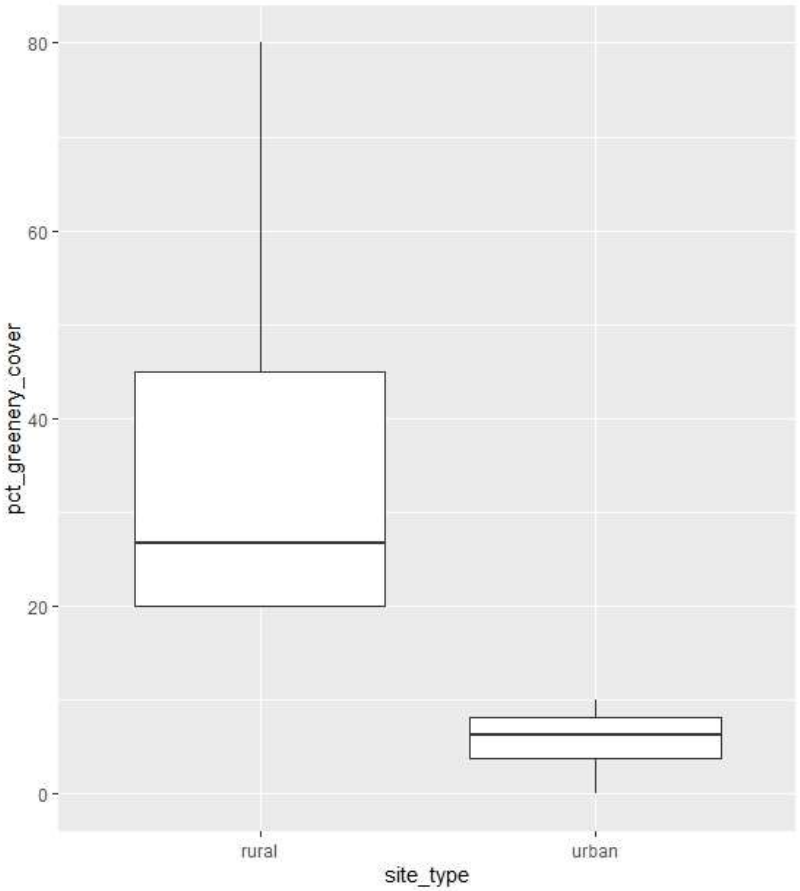

Figure A.2 Percent Green Cover at Rural and Urban Sites. Rural sites had higher percentage of green cover compared to urban sites.

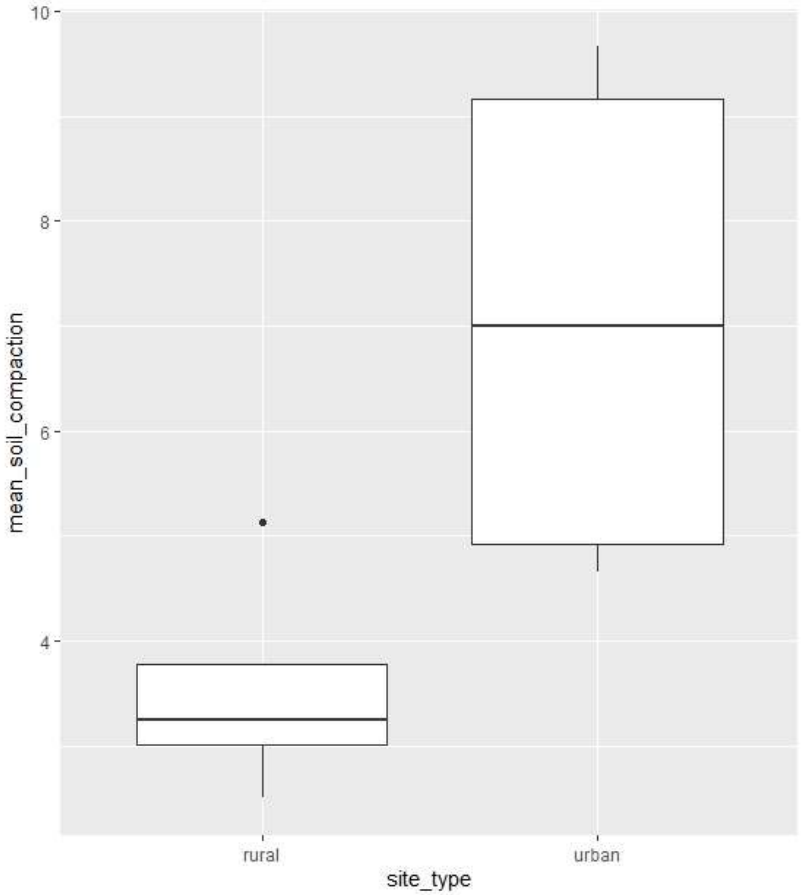

Figure A.3 Average Soil Compaction at Rural and Urban Sites. Urban sites had higher average soil compaction compared to rural sites.

Table A.4 Summary of Reciprocal Transplant Soil Heavy Metal Analysis

Element	Site type	Mean (μg/g)	SE (μg/g)	SD (μg/g)	Min (μg/g)	Max (μg/g)
Al	rural	7825.25	± 673.01	1526.38	5784.14	9243.41
	urban	6119.27	± 685.08	812.66	5289	7225.67
As	rural	7.20	± 1.60	3.99	3.14	11.63
	urban	7.77	± 1.24	2.77	5.83	11.87
Cd	rural	0.42	± 0.06	0.08	0.29	0.48
	urban	0.44	± 0.08	0.17	0.28	0.62
Co	rural	5.98	± 1.15	2.67	3.76	9.85
	urban	4.61	± 0.56	1.04	3.07	5.39
Cr	rural	25.55	± 2.85	5.63	21.51	33.87
	urban	25.52	± 7.38	15.48	15.87	48.63
Cu	rural	149.26	± 81.04	151.77	41.77	367.91
	urban	58.42	± 13.52	16.55	41.49	81.16
Fe	rural	16593.59	± 1374.43	2833.22	13550.45	19069.46
	urban	13887.35	± 1055.24	1913.82	12168.12	16169.01
Mg	rural	2597.99	± 748.11	1007.26	1363.89	3828.62
	urban	3936.64	± 1167.31	2548.10	1405.86	6670.70
Mn	rural	304.64	± 43.91	69.15	218.27	385.44
	urban	296.71	± 53.60	112.355	209.88	454.05
Na	rural	576.66	± 174.42	306.285	226.60	850.24
	urban	690.81	± 226.94	381.63	120.81	928.40
Pb	rural	172.36	± 38.30	88.38	83.77	293.26
	urban	610.74	± 477.04	782.19	137.03	1780.02
Se	rural	0.55	± 0.315	0.68	0	1.54
	urban	0.42	± 0.35	0.56	0	1.19
V	rural	30.54	± 6.08	14.97	20.94	52.79
	urban	22.29	± 4.12	6.96	14.62	31.49
Zn	rural	541.91	± 321.20	747.43	96.34	1659.04
	urban	211.88	± 33.87	57.56	149.34	268.35

Note: There are a total of 8 sites: 4 urban sites and 4 rural sites

APPENDIX B

FLOWERS AND SEED PODS

The raw data is shown for maximum flower number (Figure B.1) and seed pod number (Figure B.2).

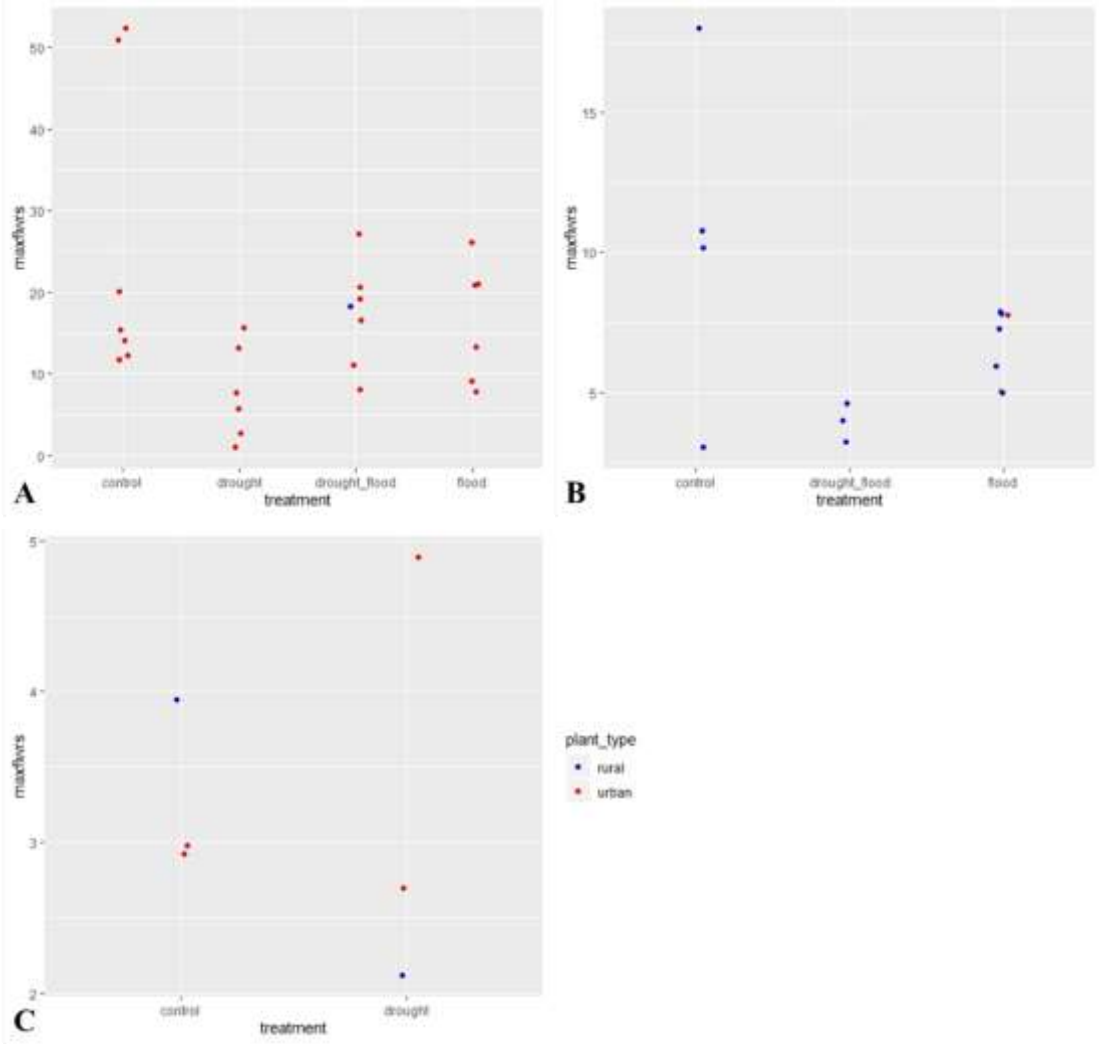

Figure B.1 Flowers Produced (raw data). Very few other plants produced flowers (12.6% across all trials). Among the three trials, (A) 22% of plants flowered (26/120) in trial one, (B) 7.6% of plants (14/184) in trial two, and (C) 10% of plants (6/60) in trial three. (A) In trial one, plants flowered in each treatment, all of which were urban plants except for one rural plant that flowered in the drought/flood treatment. (B) Conversely, the majority of plants that flowered in trial two were rural, the exception being two urban plants that flowered in the flood treatment. No plants flowered in the drought treatment of trial two. (C) Trial three had two urban plants and one rural plant produce flowers in both the control and drought treatments.

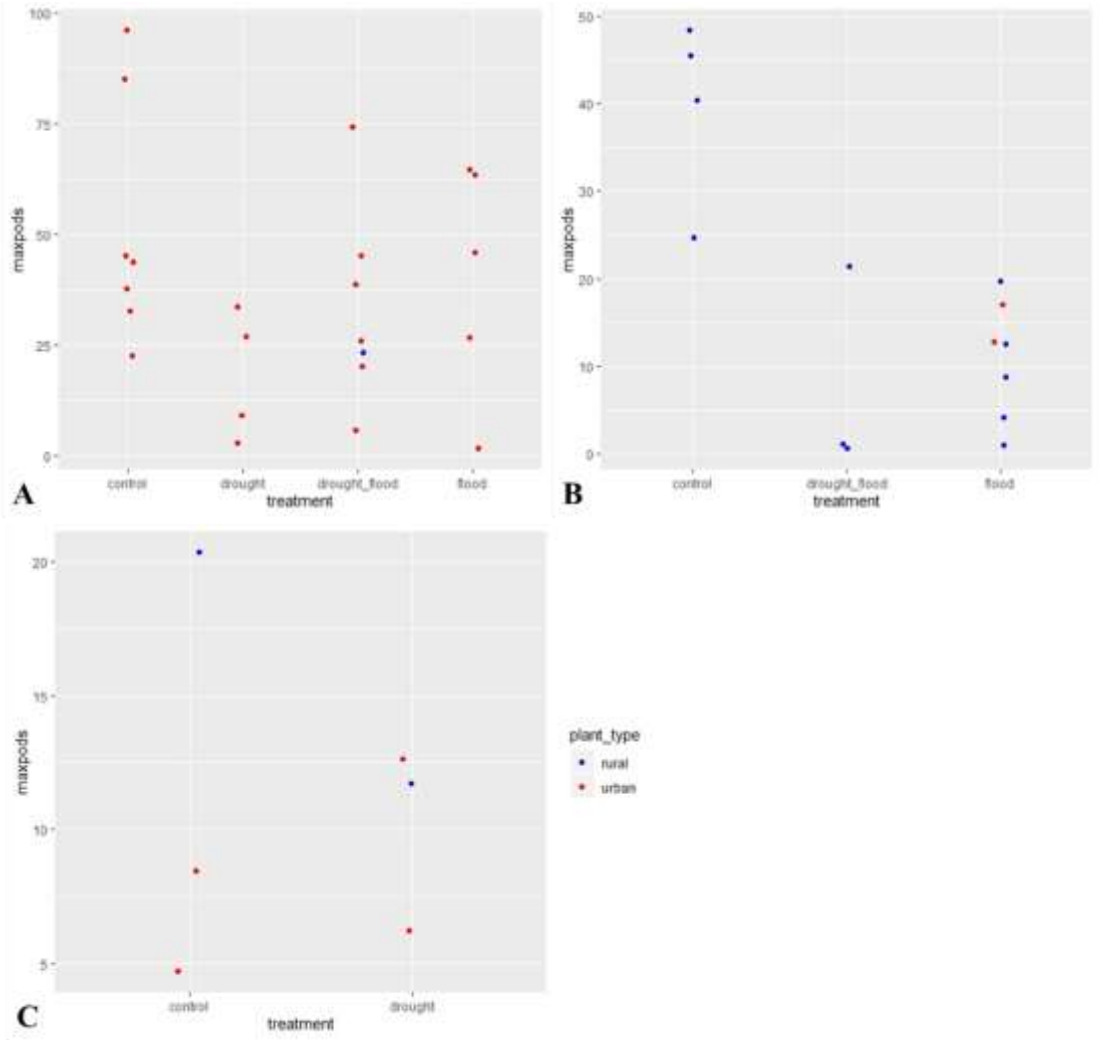

Figure B.2 Seed Pods Produced (raw data). All plants that flowered in (B) trials two and (C) three produced seed pods, as did the majority (88.5%) of flowering plants in (A) trial one when trials ended.

APPENDIX C

STOMATA AND FINAL SIZE

Example of microscope slide used for stomatal density counts is shown in Figure C.1.

Raw data of final plant size is shown in Figure C.2.

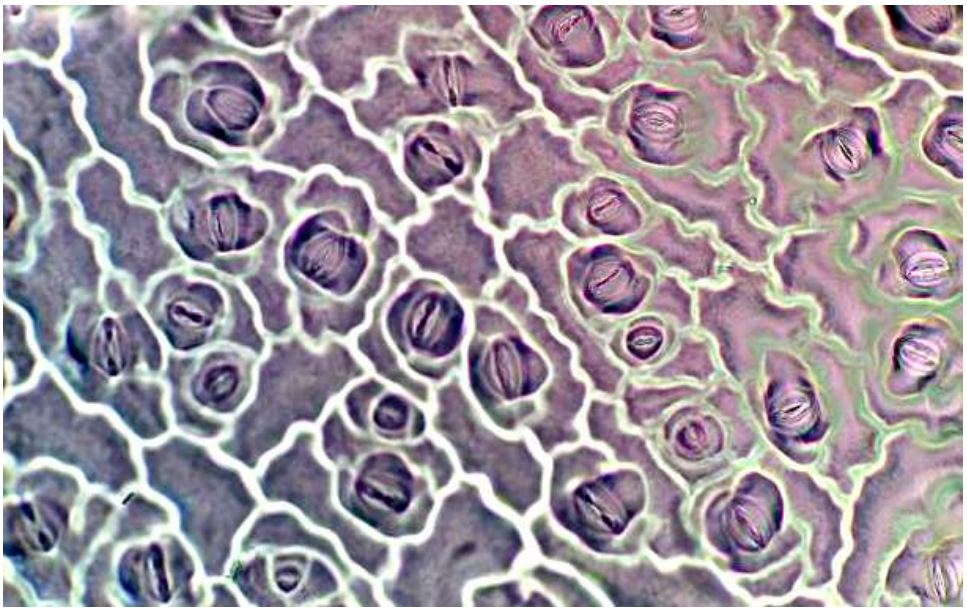

Figure C.1 Example of microscope slide used for stomatal counts. This leaf is from population 4, parent plant 53 (40x). Image is cropped with contrast increased to improve detail

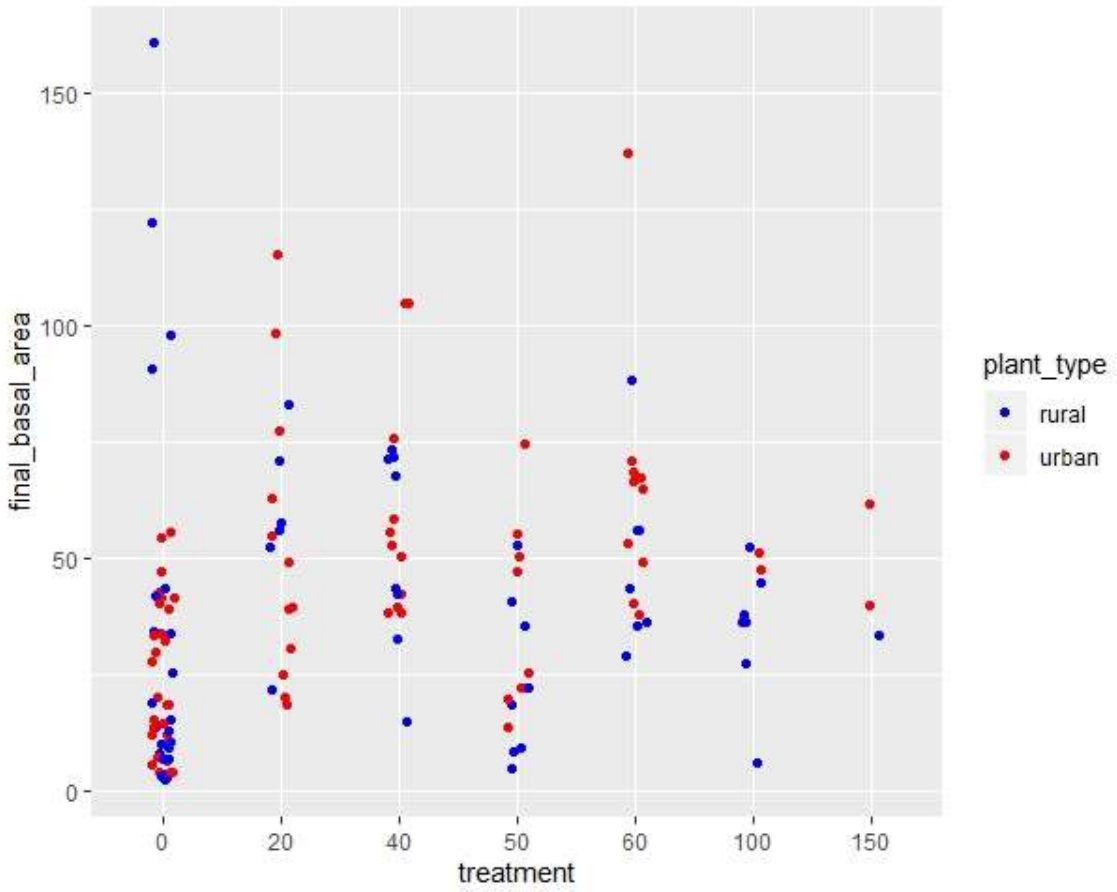

Figure C.2 Final plant size for plants that survive the salt treatments (raw data)

APPENDIX D

STOMATA AND STRESS RESPONSE GENES

Figure D.1 shows the relationship between stomatal development and certain stress response pathways.

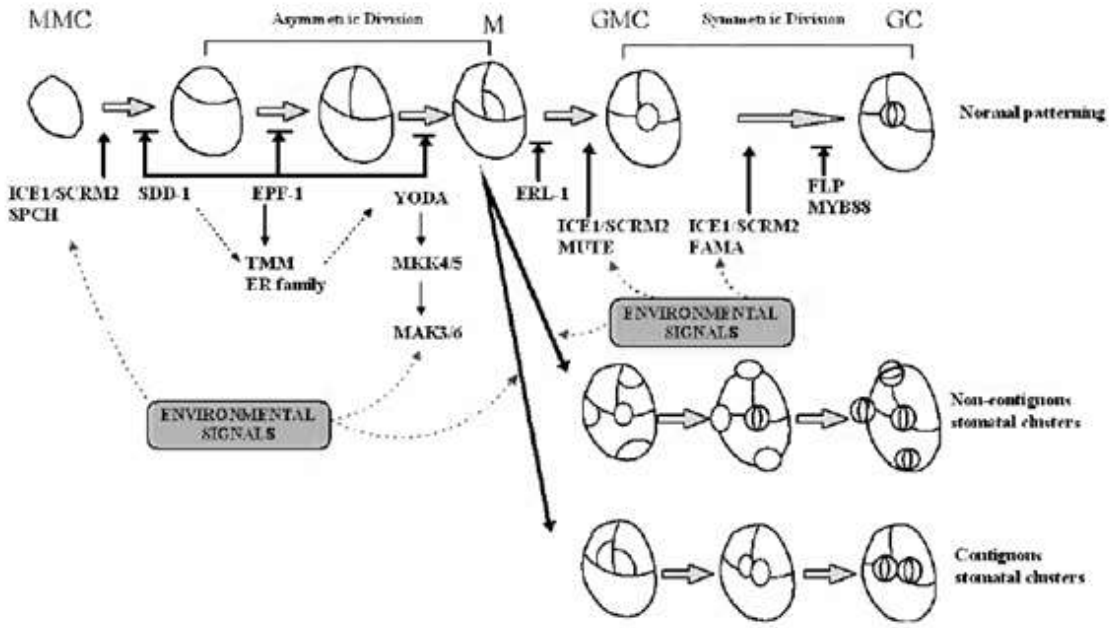

Figure D.1 "Several genes and transcription factors regulate the stomatal lineage in *Arabidopsis thaliana*. Many of them, such as YODA, MKK4/5, MAK3/6 and ICE/SCRM2, are correlated with multiple biotic/abiotic stress responses. Mutation of those genes and transcriptional factors causes excessive formation of meristemoid adjacent to the existing stoma and lead to contiguous stomatal clustering. On the other hand, the formation of non-contiguous stomatal clusters may initiate from the generation of new satellite meristemoid around the existing stoma. This pattern of cell differentiation may be modulated by environmental signals, also." (Figure 3 and legend from Gan et al., 2010) MMC = meristemoid mother cell; M = meristemoid; GMC = guard mother cells; GC = guard cell

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