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Variation in sex allocation and its implications for components of fitness in a perennial hermaphroditic plant

Chen Kai-Hsiu

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Faculté de biologie
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Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Kai-Hsiu CHEN

Biologiste diplômé du Master de l'Université de Lausanne

Jury

Prof. Philipp Engel, Président

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Variation in sex allocation and its implications for components of fitness in a perennial hermaphroditic plant

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pour le Doyen
de la Faculté de biologie et de médecine

Prof. Tadeusz Kawecki

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It is an honor and a humbling experience to pursue scientific research in a world where education gaps still exist. This has inspired me to explore further and pay it forward.

ABSTRACT

Hermaphroditic perennial organisms that produce both female and male sexual organs should allocate their reproductive resource into their two sex functions, i.e., their sex allocation, such as to maximize lifetime reproductive success in the face of potential trade-offs among survival, growth, and reproduction. In this thesis, I addressed several key questions related to sex allocation strategies and their fitness implications in wild populations of the perennial protogynous herbaceous plant, *Pulsatilla alpina* (Ranunculaceae).

By considering both the life history and sexual selection, I found that the species possesses a size- and time-dependent sex allocation strategy, characterizing it as both gender-diphasic and andromonoecious. Small individuals usually produce only one or few early-mating male flowers, likely maximizing their male reproductive success by capitalizing on the high mate opportunity in the early season as a result of the species' protogynous phenology, whereas larger individuals allocate absolutely and proportionally more to their female function.

I explicitly examined the dependency of reproductive success on sex allocation to female and male functions, a key question in the studies of the evolution of sexual systems. The results indicate that the reproductive success through one sex depends on the sex allocation also to the opposite sex in a way that goes beyond a simple allocation trade-off. This dependency is likely the result of seed discounting or sexual interference, notably because allocation to male function increased self-fertilization and compromised female function due to strong inbreeding depression. This result thus points to an important link between the mating system and sex allocation strategy. Furthermore, the mapping of reproductive success on a fitness landscape as a function of both female and male allocation illustrates a novel and potentially powerful way to apply key notions derived from sex-allocation theory to real populations by overcoming difficulties associated with currency conversion between sexual functions.

Lastly, I found that the height of floral stalks in *P. alpina*, an ancillary trait enhancing seed dispersal by wind, is a costly trait strongly associated with female allocation. Interpretation of the costs and benefits of allocation to stalk height further help to explain the gender-diphasic and andromonoecious sex allocation strategy in this and other similar species. Moreover, the

height of stalks was under disruptive selection by pollinators and seed predators in terms of components of female reproductive success, pointing to likely context-dependent phenotypic selection.

Taken together, the results of my research demonstrate an unusual empirical examination of sex allocation theories incorporating life history, sexual selection, and mating systems together in *P. alpina*. Moreover, they provide not only adaptive explanations to the evolution of sex allocation strategies of gender-diphasy and andromonoecy but also valuable insights into the general understanding of sex allocation in hermaphroditic plants.

RESUMÉ FRANÇAIS

Les organismes pérennes hermaphrodites produisent à la fois des organes sexuels femelles et mâles, et doivent donc ajuster leur allocation sexuelle, c'est-à-dire la répartition de leurs ressources reproductives entre leurs deux fonctions mâle et femelle, de manière à maximiser leur succès reproducteur total en réponse à de possibles compromis évolutifs entre survie, croissance et reproduction. Dans cette thèse, j'ai abordé plusieurs questions clés liées aux stratégies d'allocation sexuelle et à leurs implications en termes de fitness dans les populations sauvages de la plante herbacée pérenne protogyne, *Pulsatilla alpina* (Ranunculaceae).

En considérant à la fois son histoire de vie et l'influence de la sélection sexuelle, j'ai découvert que l'espèce possède une stratégie d'allocation sexuelle qui dépend de la taille des individus et du temps, la caractérisant ainsi comme étant diphasique et andromonoïque. Les petits individus ne produisent généralement qu'une ou quelques fleurs mâles en début de période de floraison, maximisant probablement leur succès reproductif mâle en capitalisant sur le nombre élevé d'opportunités de se reproduire en début de saison du fait de la phénologie protogyne de l'espèce, alors que les individus plus grands allouent davantage de ressources à leur fonction femelle à la fois en valeur absolue et en proportion de leur allocation totale à la reproduction.

J'ai explicitement examiné comment le succès reproducteur dépend de l'allocation aux fonctions femelle et mâle, ce qui constitue une question clé dans l'étude de l'évolution des systèmes sexuels. Mes résultats indiquent que le succès reproducteur à travers un sexe dépend de l'allocation au sexe opposé d'une manière qui va au-delà d'un simple compromis d'allocation. Cette dépendance est probablement le résultat d'une forme de gaspillage de graines (seed discounting) ou de phénomènes d'interférence entre les fonctions mâle et femelle, notamment parce que l'allocation à la fonction mâle augmente l'autofécondation, ce qui compromet le succès reproducteur femelle des individus en raison d'une forte dépression de consanguinité. Ce résultat met donc en évidence un lien important entre le système de croisement et la stratégie d'allocation sexuelle. De plus, la projection du succès reproducteur sur un paysage de fitness comme une fonction de l'allocation aux fonctions mâle et femelle illustre une nouvelle et potentiellement puissante manière d'appliquer des notions clés dérivées de la théorie de l'allocation sexuelle à des populations réelles, surmontant ainsi les difficultés associées à la conversion entre les unités de ressources pertinentes pour chaque sexe.

Enfin, j'ai montré que la hauteur des tiges florales chez *P. alpina*, un caractère secondaire favorisant la dispersion des graines par le vent, est un caractère coûteux fortement associé à l'allocation à la fonction femelle. L'interprétation des coûts et des bénéfices associés à la hauteur des piges florales produites aide à expliquer la stratégie d'allocation sexuelle diphasique et andromonoïque chez cette espèce et d'autres espèces similaires. Par ailleurs, j'ai démontré que la hauteur des tiges est sous sélection disruptive du fait des interactions avec les pollinisateurs et les consommateurs de graines en termes des différentes composantes du succès reproducteur femelle, ce qui indique que la sélection sur ce phénotype dépend probablement du contexte écologique.

En conclusion, mes recherches constituent un examen empirique inhabituel des théories d'allocation sexuelle incorporant l'effet de l'histoire de vie, de la sélection sexuelle et du système de croisement chez *P. alpina*. Mes résultats fournissent non seulement des explications adaptatives à l'évolution des stratégies d'allocation sexuelle diphasiques et de l'andromonoécie, mais aussi des observations précieuses pour notre compréhension générale de l'allocation sexuelle chez les plantes hermaphrodites.

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GENERAL INTRODUCTION

This thesis addresses several interrelated questions pertaining to the evolution and ecology of variation in the life-history and sex allocation of perennial hermaphroditic plants. In this general introductory chapter, I first briefly lay out the conceptual and empirical background to the questions posed, then I introduce the model system I chose to study, and I finally set out the individual chapter aims. Note that because each chapter begins with a more expansive introduction to the material covered in it, my aim in this general introduction is to provide a broad overview of the topics and to explain how the different chapters of the thesis relate to the overarching concerns of the thesis and to one another.

Conceptual and empirical background

In general, hermaphroditic organisms that produce both female and male sexual organs in the same individual should make decisions on how to allocate their resource of reproduction into the two sex functions, i.e., sex allocation, to gain reproductive success in a way that the lifetime fitness is maximized under potential trade-offs among survival, growth, and reproduction (Charnov et al., 1976; Charlesworth and Charlesworth, 1981; Charnov, 1982; Lloyd, 1984a; Charlesworth and Morgan, 1991; Zhang and Jiang, 2002). Thus, how hermaphroditic organisms adopt different sex allocation strategies and how they determine female, male, and total reproductive success are two key questions to understand different sex allocation decisions in hermaphroditic plants and animals.

Sex allocation theory developed in the 1980s has been a powerful conceptual tool to understand the evolution of sex allocation strategies and sexual systems, especially in plants (Charlesworth and Charlesworth, 1981; Charnov, 1982; Lloyd, 1984a). The classic models are usually based on an assumption that a certain amount of resources for reproduction is allocated to female and male functions under a strictly linear trade-off expressed in relative allocation. Under a such trade-off in sex allocation, natural selection should favor the allocation strategy that maximizes the total gain in reproductive success via the two sexual functions, whereas the shape of the dependency of female and male reproductive success on the relative sex allocation, so-called ‘fitness gain curves’, determines the evolutionary stable strategy (ESS) of the organism (Charlesworth and Charlesworth 1981; Charnov 1982; Lloyd 1984; see Figure 1 for an illustration of the classic model). Over the past decades, sex allocation theory has stimulated fruitful discussions and a fertile field of both theoretical and empirical studies.

Because the reproductive success of female and male functions is thought to be generally limited by resources and mates, respectively (Bateman, 1948), a series of theoretical works further addressed how variation in resource status and mating opportunity may affect sex allocation strategies in hermaphroditic plants (e.g., de Jong and Klinkhamer 1994; Brunet and Charlesworth 1995; Seger and Eckhart 1996; Diggle 1997; Zhang and Jiang 2002; Sakai and Sakai 2003; Cadet et al. 2004; Harder et al. 2004; Thomson 2006; Austen et al. 2015)

For instance, much attention from theoretical works has been put on the effects of plant size on allocation decisions. Size is highly correlated with resource status and thus a plant's ability to produce and disperse its pollen and/or seeds (de Jong and Klinkhamer, 1994; Seger and Eckhart, 1996; Zhang and Jiang, 2002; Sakai and Sakai, 2003; Cadet et al., 2004; Sato, 2004). A general prediction from the models is that a hermaphroditic plant should gradually increase its allocation towards the sex function that shows a less saturating relationship with size, resulting in a size-dependent sex allocation strategy (Klinkhamer et al., 1997; Cadet et al., 2004). For example, male reproductive success is generally thought to be saturating quicker than the female one in insect-pollinated species, thus insect-pollinated plants are predicted to show a size-dependent sex allocation with increasing female allocation (examples reviewed in de Jong and Klinkhamer 1989).

On the other hand, several studies have also focused on how changes in mating opportunity as a result of dichogamy, ontogeny, and pollinator behavior may cause different sex allocation strategies in hermaphroditic plants, especially the male allocation, among and within individuals (Brunet and Charlesworth, 1995; Diggle, 1997; Harder et al., 2004; Thomson, 2006; Austen et al., 2015). For instance, many plants separate their female and male functions in time within a hermaphroditic flower, a phenomenon called dichogamy, as a strategy to avoid selfing and/or sexual interference (Webb and Lloyd, 1986). In species where the female function precedes the male function, so-called protogyny, there are likely much more flowers at their female stage at the beginning of the season within a population. As a result, selection should favor a time-dependent sex allocation strategy that increases the male allocation in early-season flowers (Brunet and Charlesworth 1995; examples reviewed in Austen et al. 2015).

Although it is evident that the sex allocation strategies plant species adopt are likely a joint response to the selection imposed by their life history and sexual selection regimes, both theoretical and empirical works have tended to consider them independently. As a result, we are largely ignorant about how life history and sexual selection may together shape the sex allocation strategy and determine the reproductive success in a hermaphroditic plant.

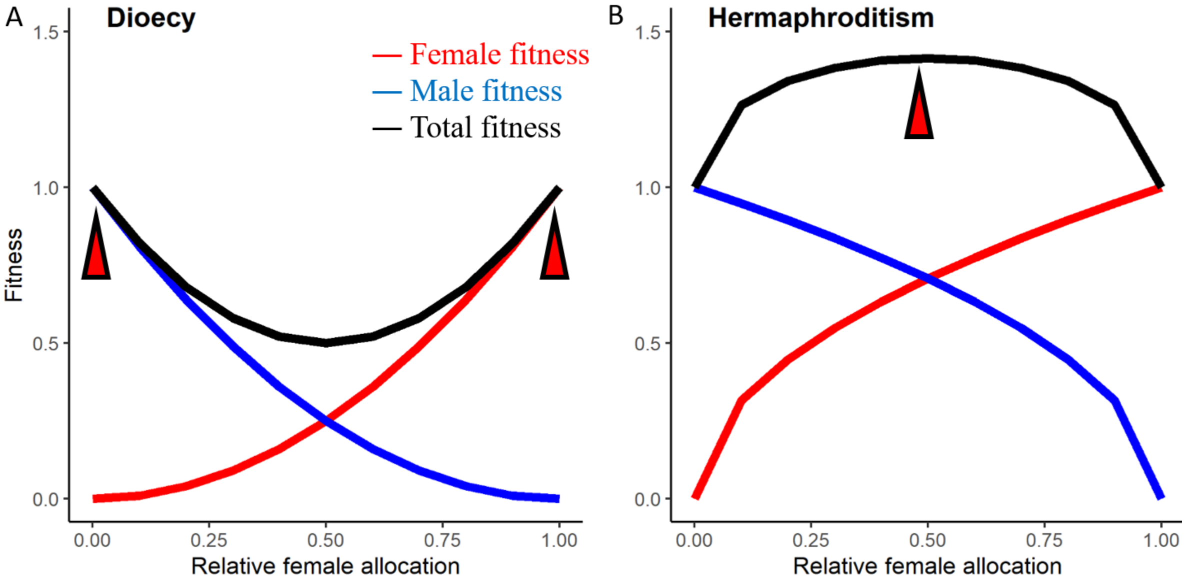


Figure 1. Conceptual illustration of the classic model of sex allocation theory (Charnov, 1982). In a monocarpic organism, a certain amount of resources dedicated to reproduction has to be allocated to female and/or male functions under a linear trade-off, which is described by the relative allocation to the female function. Fitness gain curves depict the dependency of fitness of one sex function on the relative sex allocation to the sex function (red and blue lines for female and male function, respectively). Total fitness as a sum of the fitness gained via the two sex functions is depicted by black lines. Red triangles indicate the sex allocation decision which gives the highest total fitness and by which the marginal fitness gains are equal via the two sex functions. The shape of the gain curves thus determines the stable sex allocation strategy of the organism. (**A**) The fitness gain curves of female and male functions are both accelerating. The sexual system is stable at dioecy. (**B**) The fitness gain curves are both saturating. The sexual system is stable at hermaphroditism. Different shapes of the gain curves thus lead to different stable sex allocation strategies.

Moreover, empirical examinations of sex allocation theories lag behind the development of theoretical works due to numerous challenges and limitations (see detailed discussions in Campbell 2000; Schärer 2009). In brief, it is practically challenging to find sufficient natural variation in phenotypes with different sex allocation, to define the linear trade-off between the two sex functions in a common currency, and to precisely and accurately measure reproductive success, especially for the male function (Goldman and Willson, 1986; Emms, 1993; Campbell, 2000; Thomson, 2006). As a consequence, very few studies have convincingly demonstrated the fitness gain curves, i.e., the dependency of reproductive success on sex allocation to a sex function (reviewed in Campbell 2000). Moreover, using genetic markers to estimate paternity, the latest studies investigated how plant size and male allocation affect male reproductive success examining predictions of the size-dependent sex allocation theory in wind-pollinated species, e.g., *Ambrosia artemisiifolia* (Nakahara et al., 2018; Aljiboury and Friedman, 2022) and *Mercurialis annua* (Tonnabel et al., 2019).

Nonetheless, empirical studies predominantly adopted a univariate point of view derived from the concept of fitness gain curves in the theories and assumed that the dependency is only affected by factors operating on that sex function (Charlesworth and Charlesworth 1981; Charnov 1982; Lloyd 1984; Figure 1). In the past few decades, much attention has been put on different ecological factors that may cause local resource competition, e.g., as a result of passive seed dispersal (Campbell, 2000; Takahashi et al., 2005), and local mate competition, e.g., as a result of the behavior of insect pollinators (Brunet, 1992; Campbell, 1998), and thus lead to a saturating gain curve in the female and male function, respectively (Charnov et al., 1976; Charlesworth and Charlesworth, 1981). On the other hand, animal-dispersal and wind-pollination may decrease the competition among related seeds and pollens, thus leading to accelerating or linear gain curves (Charnov, 1979; Charlesworth and Charlesworth, 1981; Burd and Allen, 1988).

We are largely ignorant about the fact that the reproductive success of one sex function may depend not only on the allocation to itself but also on that to the opposite sex and go beyond a simple allocation trade-off assumed in the theories. First of all, the selfing rate of the female function is likely to depend on the sex allocation, especially the male function, which may lead to a reduction in female reproductive success with the presence of inbreeding depression, so-called ‘seed discounting’ (Lloyd, 1992). In a theoretical work by de Jong et al. (1999), different scenarios of dependency of the selfing rate on male allocation and different degrees of inbreeding depression were shown to have various implications on the ESS of sex allocation strategy, e.g., from size-dependent sex allocation strategy to dioecy. Moreover, it is plausible that the two sex functions may interfere biochemically or physically and affect the reproductive success of each other within a hermaphroditic individual or flower (Barrett, 2002), leading to a dependency of reproductive success on the allocation to the opposite sex function. Take the andromonoecious *Solanum carolinense* for example, the species produces male and hermaphroditic flowers that differ qualitatively in their female sex allocation, it has been suggested that female sex allocation in hermaphroditic flowers interferes physically with pollen removal by bumble bees, reducing their male reproductive success, a problem obviated in flowers with only male function (Elle and Meagher, 2000). Despite the high likelihood that sex allocation to one sex affects the reproductive success of the opposite sex, the latest empirical studies still considered the dependency of reproductive success on sex allocation from a one-dimensional point of view (e.g., Nakahara et al. 2018; Tonnabel et al. 2019; Aljiboury and Friedman 2022). To my knowledge, no empirical study has yet explicitly investigated the dependency of reproductive success on sex allocation to both sexual functions from a two-dimensional point of view with the effects of the mating system taken into account. This points to a gap in the field that needs careful investigation.

The fulfillment of reproductive success depends on the investment in producing not only female and male organs but also ancillary traits that facilitate reproduction (Friedman and Harder, 2004; Vittoz and Engler, 2007; Fleming and John Kress, 2011), though the cost and fitness implications of the latter are largely overlooked in studies of sex allocation. The majority of plant species rely on biotic or abiotic vectors to transmit their pollen with the aid of various ancillary traits (Proctor et al., 1996; Ollerton et al., 2011). These ancillary traits usually require a substantial investment of resources, implying a cost, and facilitate the reproductive success of either one or both of the sexes, implying a benefit. On the one hand, for instance, showy petals and copious nectar rewards may serve as an attractant for animal pollinators (Schiestl and Johnson, 2013; Moyroud and Glover, 2017), which is likely to benefit both sexes. Whereas elongated stalks or peduncles may facilitate pollen dispersal by wind and thus enhances male reproductive success (Friedman and Harder, 2004; Friedman and Barrett, 2009). On the other hand, ancillary traits such as fleshy fruits and elongated fruit stalks may enhance seed dispersal distance by animals and wind, respectively, and thus facilitate female reproductive success (Howe and Smallwood, 1982; Soons et al., 2004; Fleming and John Kress, 2011; Palacio et al., 2023). Despite the importance of ancillary traits on the benefits of reproductive success and the cost of resource allocation, we are largely ignorant about how they may affect the evolution of sex allocation strategies and sexual systems.

The expression of a trait is usually dynamic over different developmental stages and may affect reproductive success via various components in different directions. For instance, it has been found that the height of stalks of males of wind-pollinated, dioecious *Rumex hastatulus* was greater than that of females during flowering, but that female stalk height was greater during fruiting and seed dispersal (Pickup and Barrett, 2012), which likely reflects the selection operating on pollen dispersal and seed dispersal, respectively. Furthermore, the association of a trait and reproductive success may be context-dependent, as the same trait may be expressed through multiple life stages interacting with various agents (e.g., not just mutualists but also antagonists), and the net selection of the trait via one sex function depends on the net outcome of all those components (Gómez, 2008; Ågren et al., 2013). For example, in a classic study in *Erysimum mediohispanicum*, Gómez (2008) showed evidence of conflicting selection imposed by pollinators, seed predators, and/or abiotic factors on stalk height and flower diameter via quantifying the selection in different female fitness components over various life stages. As a result, the study stresses that a complete interpretation of ancillary traits facilitating sexual functions necessitates a detailed examination of their effects on reproductive success through various stages.

In this thesis, I investigate the following three general questions, using the protogynous perennial herb *Pulsatilla alpina* as a model:

1. How do hermaphroditic organisms adjust their sex allocation to their female and male functions as a response to resource status and the opportunity of mating? What are the fitness implications of such a sex allocation strategy?
2. How does reproductive success depend on sex allocation to both female and male functions? Does inbreeding depression affect that dependency?
3. How do ancillary traits affect different fitness components of one sex function? What is its implication on sex allocation strategy?

Study system and study sites

Pulsatilla alpina (L.) Delarbre (Ranunculaceae) is a perennial, protogynous, andromonoecious hemicryptophyte that grows in sub-alpine to alpine habitats in central Europe (Lauber et al., 2018). Several vegetative and/or reproductive shoots emerge from a perennial underground rhizome soon after the snowmelt, from early May to July. The plants vary greatly in both aboveground size (number of leaves and flowers) and in the size of the persistent underground rhizome. Individuals produce up to approximately twenty white flowers, each on its reproductive shoot (Figure 2D). Phenotypically male flowers bear only stamens (Figure 2C), whereas protogynous hermaphroditic flowers bear stamens and uni-ovulate pistils (Figure 2A and B). In the populations studied here, stamen and pistil numbers varied between approximately 150 and 400, and zero and 400, respectively.

Flowers of *P. alpina* are predominantly visited by dipteran insects for pollen, including houseflies and syrphid flies (Chen and Pannell 2022; Figure 3A). Flowers of *P. alpina* are also visited by *Phytomyza* species, which are monophagous, dipteran seed predators (Figure 3B). These flies mate in the flower, the female adults oviposit on the pistils, and the larvae eat the pollinated ovules during the fruiting stage, as found for other *Phytomyza* species that eat seeds within the achenes (Winkler et al., 2009). Ripe achenes with elongated pappus hair are dispersed by wind in early autumn (Vittoz and Engler 2007; Figure 3C). After the achenes are dispersed in late autumn, the above-ground vegetative parts of individuals die away, but individuals persist as rhizomes below ground until the next spring. Individuals of *P. alpina* are subject to above-ground herbivory mainly as a result of direct consumption or trampling by cattle, usually in late summer, which may affect the resource status and thus the sex allocation of individuals.

The results presented in this thesis are the fruits of observations and experiments conducted in 13 populations of *P. alpina* in the pre-Alps of Canton Vaud, Switzerland, over five consecutive years from 2018 to 2022 (see Appendix S2 of **Chapter III** for the details of each population). The populations vary in population size (from around a hundred to more than a thousand individuals), elevation (from 1,694 to 2,122 m a.s.l.), flowering season (mid-May to early July), herbivory intensity, and sex allocation of individuals, thus it is an ideal system to address

Figure 2. Photographs of a female-stage (**A**) and male-stage (**B**) hermaphroditic flower, a male flower (**C**), and a relatively large individual with multiple flowers (**D**). In hermaphroditic flowers, the female stage precedes the male stage, i.e., protogyny.

various questions regarding the ecology and evolution of sex allocation strategies within and among populations.

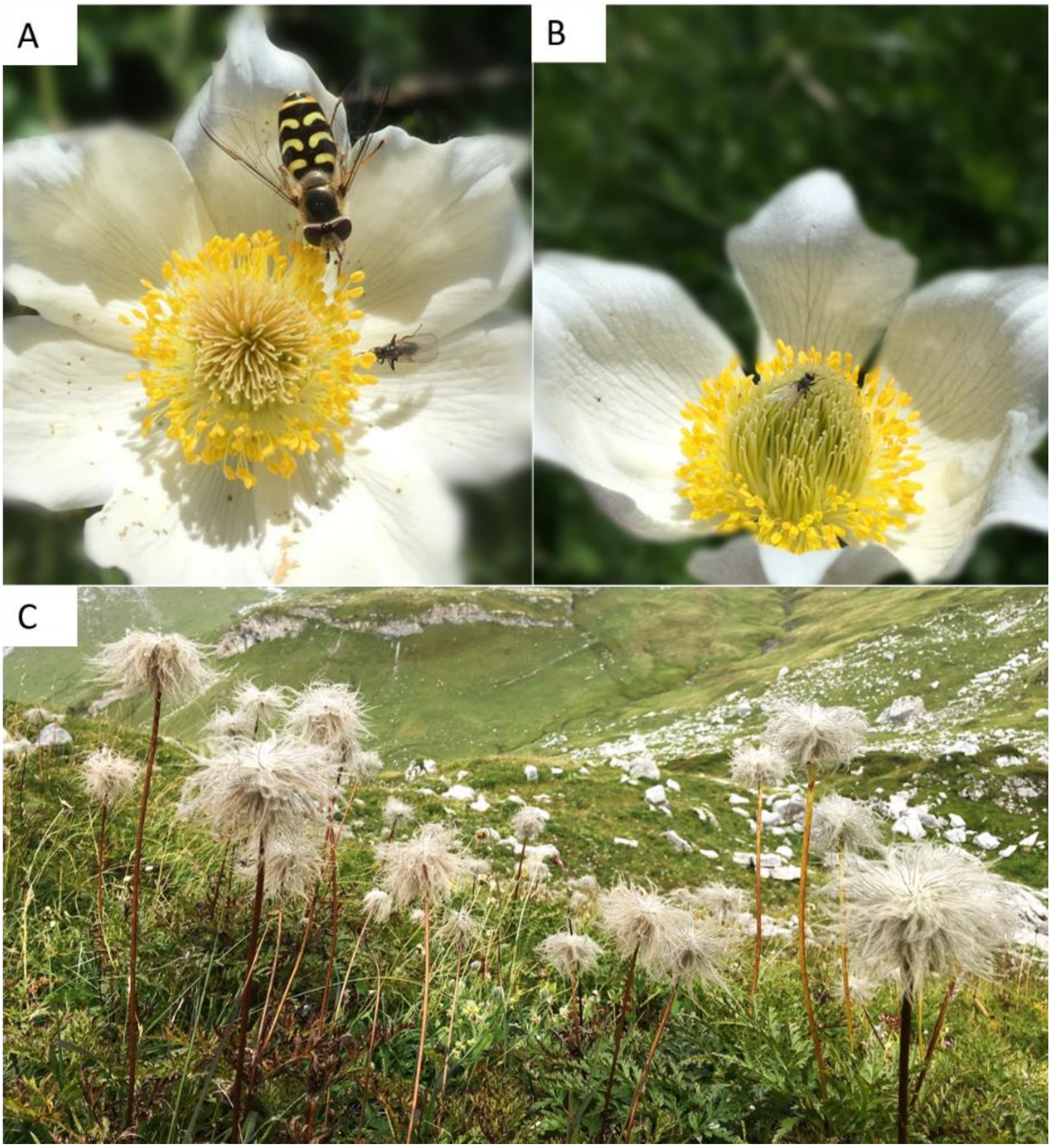


Figure 3. Photographs of dipteran insects visiting a hermaphroditic flower of *P. alpina* for pollen (**A**), a female adult of the pre-dispersal seed predator, *Phytomyza* sp., ovipositing on the pistils of a hermaphroditic flower (**B**), ripen achenes on elongated stalks at the end of the growing season ready for dispersal by wind in the alpine grassland (**C**).

Thesis outline

My thesis is divided into three main chapters. In **Chapter I**, I aim to examine how sex allocation and seasonal reproductive success of female and male functions depends on both intrinsic resource status and extrinsic mating environment at the individual level, i.e., the two factors that are thought to govern female and male reproductive success, respectively, in the perennial and protogynous herb. I specifically aim to quantify male reproductive success by conducting a detailed phenology record throughout a season to estimate prospective siring success. I ask whether the species adopts a sex allocation strategy predicted by sex allocation theories considering plant size and timing of flowering independently in hermaphroditic plants and whether consideration of the two factors of both life history and sexual selection jointly provides new insights on strategies of gender-diphasy and andromonoecy.

In **Chapter II**, I focus on how sex allocation determines reproductive success at the flower level via a paternity analysis using a population comprising mostly single-flowered individuals of *P. alpina*. The study aims to first quantify how sex allocation affects the mating system and thus reproductive success by taking into account inbreeding depression. Secondly, I adopt different approaches to mapping reproductive success to sex allocation from the conventional concept of one-dimensional ‘fitness gain curves’ to three-dimensional ‘fitness landscapes’. These complimentary approaches allow us to examine whether there is a dependency of reproductive success on the sex allocation of the opposite sex, likely as a consequence of sexual interference, and whether the interaction between the two sex functions leads to sophisticated fitness landscapes of total reproductive success at the flower level that could not be revealed by simple assumptions of fitness gain curves.

In **Chapter III**, I focus on an ancillary trait, the height of floral stalks, which shows a strong positive correlation with the female sex allocation of a flower. I adopt different approaches in the field to study natural variation among populations, development of the trait, implications on the cost of such trait, and phenotypic selection in female reproductive success via mutualistic and antagonistic biotic interactions. By drawing the results from these approaches, I aim to synthesize a general understanding of how the stalk height may affect the sex allocation strategy of the species.

CHAPTER I:

Size-dependent sex allocation and the expression of andromonoecy in a protogynous perennial herb: both size and timing matter

Kai-Hsiu Chen and John R. Pannell

(under review in *American Naturalist*)

Abstract

The optimal life history and sex allocation of perennial hermaphrodites should depend on both their size and the relative costs and benefits of reproducing through male versus female functions. Theory predicts that insect-pollinated perennials should increase their allocation to female function with size, while the ‘mating environment’ hypothesis predicts that allocation to male function should track mating opportunities over the course of flowering. We test these two predictions by inferring male and female reproductive success in the protogynous perennial herb *Pulsatilla alpina* by tracking the patterns and dynamics of sex allocation over time for marked individuals over a range of sizes. We found that small individuals tend to produce only male flowers and that both small and larger individuals produced male flowers at the beginning of the flowering season when mating opportunities were high. By considering within-population variation in life history and phenology jointly rather than separately, and by considering both the tradeoff costs and benefits of allocation to male versus female functions, our results provide new insights into the evolution of both gender diphasy and andromonoecy in perennial plants that are constrained by a dichogamous flowering strategy.

Keywords: Environmental sex determination, Tradeoff, Gender diphasy, Plasticity, Dichogamy, Paternity

Introduction

Perennial hermaphroditic plants face a complex set of allocation decisions that affect their life history and sex allocation – the two principal axes of reproduction. First, they must determine when, and at what size, to begin to flower, and whether then to invest in reproduction more heavily in some years over others (aspects of their life history) (Lovett Doust, 1989; Wenk and Falster, 2015; Roach and Smith, 2020). Second, they must determine how much of their reproductive resources to allocate to their male versus female sexual functions (their sex allocation, ‘SA’) (Charnov, 1982; Charlesworth and Morgan, 1991). Life-history and SA decisions are each important on their own, but they should also be linked because tradeoffs between growth and reproduction will often differ through reproduction through male versus female functions (Iwasa, 1991; Obeso, 2002; Dorken and Van Drunen, 2018). Empirical assessment of the marginal costs of male versus female function in terms of growth and survivorship indicates that plants often invest more heavily in their female function than their male function (Obeso, 2002). This is particularly evident in dioecious species in which adult sex ratios of reproducing individuals are male-biased as a result of the greater mortality or less frequent flowering of females that bear a higher cost of reproduction (Sinclair et al., 2012; Barrett and Hough, 2013; Field et al., 2013), but it is also evident in hermaphroditic species in which plants that have invested heavily in their female function are less likely to flower the following year than those that have reproduced as males (Schlessman, 1991; Zhang et al., 2014; Blake-Mahmud and Struwe, 2019; Bialic-Murphy et al., 2020).

Whereas patterns of SA in plants are often interpreted from the perspective of the relative costs of male versus female functions (Goldman and Willson, 1986; Case and Ashman, 2005), selection optimizes phenotypes in terms of the costs *and benefits* of the strategies they adopt. Thus, while a greater cost of female function has been invoked to explain patterns of size-dependent SA in which plants emphasize their male function when small and their (costlier) female function when large (Lloyd, 1984b), these patterns must also depend on the relative benefits to small versus large individuals of reproducing through male versus female function (Ghiselin, 1969; Klinkhamer et al., 1997). In insect-pollinated plants, fitness gained through allocating to male function is thought to saturate more quickly than through female allocation, so plants with a large budget should have a female-based SA (de Jong and Klinkhamer, 1989, 1994). In contrast, tall plants of wind-pollinated species may enjoy direct effects of fitness because height is expected to enhance pollen dispersal, so we might expect large size to be associated with the male function (de Jong and Klinkhamer, 1994; Sakai and Sakai, 2003; Cadet et al., 2004). Importantly, in species with wind-dispersed fruits or seeds, female fitness might also benefit from the direct effects of height (de Jong and Klinkhamer, 1994; Soons et al., 2004; Pickup and Barrett, 2012).

The relative benefits of flowering through male versus female functions may also vary over time *within* a flowering season (Brunet and Charlesworth, 1995). Because all flowers in a population do not open simultaneously, the pollen from flowers produced at different times during the flowering season will have different opportunities to sire offspring, and the intensity of male-male competition will vary accordingly; we might refer to this idea as the 'mating environment' hypothesis (Brunet and Charlesworth, 1995). In protandrous species, where the male function precedes the female function, more females will be available late in the season, and we should therefore expect some plants to capitalize on the greater availability of female mates by emphasizing their male function towards the end of the flowering season. An increase in relative allocation to male function has indeed been reported for some protandrous species (but see Aizen 2001), e.g., *Aquilegia caerulea* (Brunet, 1996), *Campanula rapunculoides* (Vogler et al., 1999), *Cimicifuga simplex* (Pellmyr, 1987), and *Corydalis ambigua* (Kudo et al., 2001). In protogynous species, by contrast, the mating environment hypothesis predicts an increased allocation of resources to male function by some individuals early in the season (Brunet and Charlesworth, 1995).

While the mating environment hypothesis provides a plausible explanation for an increase in relative allocation to male function in protandrous species late in the flowering season, alternative theories predict the same pattern. For instance, the 'resource competition' hypothesis proposes that flowers later in a season have fewer resources to draw upon due to the allocation of resources into fruiting by early-season flowers and that late flowers should avoid the costly female function and emphasize their male function (Diggle, 1994; Medrano et al., 2000). Alternatively, the 'architecture effect' hypothesis suggests that resources are more available to flowers at the base of the inflorescence, which are usually the early flowers (Wyatt, 1982; Diggle, 1995), such that an acropetal decline in allocation towards female function along the inflorescence should be expected. Significantly, although these hypotheses provide alternative explanations for within-season shifts in allocation in protandrous species, only the mating environment hypothesis predicts an increase in relative allocation to male function in early flowers of protogynous species at both the individual and flower levels (Brunet and

Charlesworth, 1995; Huang et al., 2004). As a consequence, protogynous species provide better systems to test the mating environment hypothesis of SA.

The predicted higher relative allocation to male function in early-season flowers of protogynous species has rarely been tested (Austen et al., 2015). A decline in anther number in later flowers within individuals has been found in protogynous *Aquilegia yabeana* (Huang et al., 2004) and *Helleborus foetidus* (Guitián, 2006), but these studies did not assess the temporal dynamics of mate availability and prospective male reproductive success ('RS') over the course of a flowering season (though see Gleiser et al. 2008). We also know little about how SA varies as a function of *both* size and phenology jointly. A few empirical studies have reported an extreme pattern of shifting from functionally a pure male to a hermaphroditic strategy with size (Schlessman, 1991; Kudo and Maeda, 1998; Peruzzi et al., 2012; Zhang et al., 2014; Niu et al., 2017), but we remain largely ignorant about the fitness of the functionally pure males. We are particularly ignorant of how plants respond to changing opportunities of mating within seasons, and of how the strategy they adopt depends on their size.

In this study, we ask how perennial plants respond to the changing costs and benefits of allocation to male versus female sexual functions over time between and within seasons. Specifically, we studied the size, flowering behavior, and SA of marked individuals of the strongly protogynous alpine herb *Pulsatilla alpina* (Ranunculaceae) over consecutive seasons, as well as in detail over time within a reproductive season, and we used our observations to evaluate the mating environment hypothesis on the basis of inferred male and female fitness components. We were particularly interested in seeking an explanation for (1) the expression of gender diphasy in *P. alpina*, in which small (and likely young) individuals produce only male flowers and larger (older) individuals are hermaphroditic, and (2) the evolution of the heteromorphic sexual system as andromonoecy, where plants produce purely male flowers in the context of an overall hermaphroditic SA strategy. Our study illustrates the value of a dynamic assessment of both life history and SA jointly in terms of the changing costs and benefits of mating as male versus female.

Materials and methods

Study species and study sites

Pulsatilla alpina (L.) Delarbre (Ranunculaceae) is a perennial, protogynous, andromonoecious hemicryptophyte that grows in sub-alpine to alpine habitats in central Europe (Lauber et al., 2018). Several vegetative and/or reproductive shoots emerge from a perennial underground rhizome soon after the snowmelt, from early May to July. Individuals produce up to approximately twenty white flowers, each on its own reproductive shoot. Phenotypically male flowers bear only stamens, whereas protogynous hermaphroditic flowers bear stamens and uni-ovulate pistils. In the populations studied here, stamen and pistil numbers varied between approximately 150 and 400, and zero and 400, respectively. The flowers are predominantly visited by dipteran insects, including houseflies and syrphid flies (**Chapter III**; Chen and Pannell 2022). Ripe achenes with elongated pappus hair are dispersed by wind in early autumn (Vittoz and Engler, 2007). After the achenes are dispersed in late autumn, the above-ground vegetative parts of individuals die away, but individuals persist as rhizomes below ground until the next spring.

We studied key aspects of the life history, SA, and reproductive biology of *P. alpina* in several populations in the pre-Alps of the canton Vaud, Switzerland during the flowering seasons of 2018 to 2020: one population at Les Mosses (Population LM), two populations at Lac Lioson (Populations LL1 and LL4), and two populations at Solalex (Populations S1 and S2). Population LM (625 individuals) is located on an open slope of sub-alpine grassland surrounded by forest, covering an area with dimensions of about 200 m x 50 m. Populations LL1, LL4, S1, and S2 (all > 1,000 individuals) are located on open slopes of sub-alpine grassland (see Appendix S1 for a more detailed description of the populations). In Population S1, we tracked flowering behavior and the SA of individuals over two consecutive seasons to investigate how individuals shift their SA. In Population LL1, we assessed the siring ability of pollen from male and hermaphrodite flowers (outcross and self-pollination). In Population LM, we tracked the flowering status, SA, and mating opportunities of marked individuals over the course of the flowering season to relate components of RS to plant size and the timing of reproduction through male and female functions. Finally, we measured SA and mate availability again in 2019 in Populations LL1, LL4, S1, and S2 to determine the extent to which patterns found in Population LM are similar among different populations with different size distributions.

Siring ability of pollen from different sources

In Population LL1 we conducted hand pollination treatments in 2018 to assess the ability of pollen from male and hermaphrodite flowers of *P. alpina* to sire progeny and to compare siring ability via outcrossing versus selfing. Individuals with more than one floral shoot were chosen at the beginning of the flowering season. Floral buds of hermaphrodite flowers were bagged with tea bags as soon as the floral shoots emerged until opening. Anthers in the flower were removed to avoid self-pollination. The pollination manipulations were conducted after the flower opened and before the anthers dehiscence, that is at the female stage (see Appendix S2 for details). The pistils in the flowers were hand-pollination with pollen from a flower from the same individual (selfing, N = 10), from a male flower in another individual (outcrossing, N = 10), or from a hermaphroditic flower in another individual (outcrossing, N = 12). The pollen used in outcrossing manipulations was from individuals at least 5m away from the hand-pollinated individuals. After the manipulation, the flowers were bagged again until fruiting. In addition, open-pollinated flowers were labeled as control (N = 15). In early autumn, all parts of the seed head were collected from the plants before dispersal. The seed set was counted as the mature seed number divided by the total pistil number.

Phase changes in individual SA from one season to the next

To assess how the SA of individuals changes among seasons, we marked 111 individuals in Population S1in 2019 (as described above for Population LM), including in our sample both flowering and non-flowering individuals and the full range of sizes and SA. The SA at the flower and the individual levels was quantified in 2019 and 2020, as described below for Population LM.

Flowering phenology

In Population LM, we studied flowering phenology in 2018 by recording the flowering state of all 625 individuals every three or four days throughout the flowering season, from early May to late June. All flowering individuals in the population were marked with a metal tag nailed into the ground beside each plant. Flowers were individually labelled with a paper tag. For each flower, we recorded its sexual stage (female or male) at each time point from its opening until it wilted. Furthermore, we recorded the SA of each flower using four ordinal categories based on visual inspection of the pistil number, i.e., zero, one to 50, 51 to 150, and more than 150 pistils. For a subsample of 88 individuals, we also photographed each flower at the late female or early male stage to count the number of stamens and pistils (see details below). The flowering date for each flower was calculated as the mid-date between its opening and its wilting, while the flowering date for each plant was calculated as the mid-date between the opening of its first flower and the wilting of its last flower.

Plant size

In Population LM, we estimated aboveground biomass (hereafter, size) at the end of the flowering season in 2018 by harvesting all aboveground parts of a subsample of 88 of the 625 individuals in the population. The subsampled tissues were then dried in an oven for five days at 60°C and then weighed. Individuals produce new aboveground tissue each growing season from resources stored in the rhizome and root tissues. Although belowground biomass was not estimated in this study (it would have required killing the plants), belowground and aboveground biomass is typically correlated in plants (Müller et al., 2000; Enquist and Niklas, 2002).

Estimates of SA and functional gender

In Population LM, we further counted the number of stamens and pistils produced in each flower, estimated the absolute biomass allocated to each sex, and used these data to calculate the functional gender for each of the 88 individuals sampled (Lloyd 1980; see below). To estimate female allocation, we collected and counted all pollinated and unpollinated pistils in each flower at the end of flowering from July to August (see estimates of seasonal female RS below). To estimate male allocation, we counted stamens photographed on each flower at the time of harvest. To account for potential biases in counting floral parts on the basis of photographs rather than the samples themselves, we counted the number of stamens directly on 15 flowers and determined the relation between the two counting methods using regression, which was: $m_R = 1.66m_P + 27.2$ $(r^2 = 0.654)$, where m_R and m_P are the estimates of stamen number on the basis of direct and image-based counts, respectively. We calculated the absolute allocation to male and female functions by multiplying the number of pistils and stamens by the mean weight of one pistil (4.41×10^{-4} g, estimated by averaging over 108 pistils) and one stamen (6.82×10^{-5} g, estimated by averaging over 770 stamens).

We estimated the functional gender (femaleness) at the individual level for all 625 individuals in Population LM based on the ordinal allocation categories mentioned below, and for all 88 sampled individuals based on actual counts of pistils and stamens. For both these estimates, we calculated individual functional gender according to Lloyd (1980) as

$$G_i = \frac{f_i}{f_i + Em_i},$$

where f_i is the number of pistils, m_i is the number of stamens produced by the ith individual, and $E = \sum f_i / \sum m_i$ is an equivalence factor that accounts for the fact that the total number of genes transmitted through male and female functions must be equal at the population level. For the full sample of 625 individuals whose allocations were estimated in terms of ordinal allocation categories of the flowers produced, we determined f_i as 0, 25, 100, and 225 pistils for the four ordinal categories, respectively, and m_i as 204 stamens for all of the four sex-allocation categories, as we did not find any difference in the stamen number between male and hermaphroditic flowers. We then used these data to characterize the gross distribution of gender throughout the population. In addition, we used the more detailed data from the 88 subsampled individuals to relate the gender of plants to their size and flowering time during the season.

Estimates of seasonal female RS

To estimate the seasonal RS via female function in Population LM (hereafter, female RS), we collected the achenes produced in each flower at the end of the growing season, using the same sample of 88 individuals measured for size and SA described above. We separated achenes into immature and mature categories and weighed. The immature achenes included non-fertilized pistils and achenes that had been damaged (killed) by seed predators (for details, see **Chapter III**; Chen and Pannell, 2022). We then estimated the female RS as the number of mature achenes produced.

Estimate of seasonal male RS

To infer the prospective seasonal male RS in Population LM, we assumed a mass-action model of mating within a series of time windows over the course of the flowering season, which is commonly used to estimate the male RS in large wild populations in which estimates based on genotyping is unrealistic (e.g., Policansky 1981; Kudo and Maeda 1998). At each of the successive time points sampled, we assumed that each individual in the population could expect to sire an equitable fraction of all the ovules available to be fertilized within that time window, with the fraction calculated as the number of mature stamens on the individual at that point in time divided by the total number of stamens across the population. We calculated prospective male RS by multiplying this fraction with the number of pistils available at that time and with the mean mature seed set of the population, which is a constant independent of time (Appendix S3). Our estimate assumed no selfing at the flower level (autogamy is likely to be minimal because the species is strongly protogynous), but we did allow for geitonogamous selfing if the male and female stages of two flowers from the same individual overlapped (at a rate determined by random mating, according to the same the mass action model assumed for all other flowers). To calculate the total prospective male RS for each individual over the flowering season, we then summed the siring success inferred for each time window over the entire flowering for that individual. We ensured that the total male and female components of RS summed over all individuals over the whole flowering season were equal, as must be the case (given that all seeds have both a mother and a father).

Patterns of mate availability and SA among populations

To determine whether the observed patterns of pistil availability and SA in Population LM (in 2018) reflect those in other nearby populations, we monitored flowering phenology, estimated SA, and measured plant size with the same methods described above in Populations LL1, LL4, S1, and S2 in 2019; these populations were larger and had a wider range of plant sizes than Population LM. In each population, we sampled around 70 flowering individuals of different sizes and labeled around 30 non-flowering individuals. The phenology of the populations and SA of the individuals were recorded once or twice a week through the flowering season, as described above. All aboveground tissue of the sampled individuals was harvested at the end of the growing season for weighing and analysis.

Statistical analysis

All statistical analysis was conducted in R version 4.0.3 (R Core Team, 2021). A generalized linear mixed model (*glmer* function in the R package *lme4* R; Bates et al., 2015) was built to evaluate the effects of pollination treatments on mature seed set. The mature seed set of each flower was set as a response variable with a binomial error distribution. Hand-pollination treatment (with four levels) was set as the fixed effect. Flower identity was set as a random effect to account for the non-independence of seeds from the same flower (the experimental unit was one seed). We used a posthoc Tukey test (*glht* function in the R package *multcomp*; Hothorn et al., 2008) to test the difference in mature seed set among treatments.

A generalized least square model (*gls* function in *nlme* package; Pinheiro et al., 2022) was built to evaluate the effects of size and flowering date on the absolute SA between female and male sex functions at the individual level, with absolute SA set as a response variable. In all analyses, size was transformed as log(size + 1) and then standardized to a mean of zero and a standard deviation of one. The flowering date was also standardized to a mean of zero and a standard deviation of one. Standardized size, standardized flowering date, and sexual function were set as the fixed effects, with three two-way and one three-way interaction terms. Variance in the absolute SA was allowed to differ between male and female functions. Individual identity was set as a random effect to account for the non-independence of absolute allocation to female and male functions within the same individual.

To evaluate the effects of size and flowering date on functional gender at the individual level, we built a linear model using the *lm* function in R, with functional gender set as the response variable and standardized size and flowering date together and their interaction set as fixed effects. To evaluate the effects of size and flowering date on female and male RS at the individual level, a *gls* model was used with the same model structure and relative RS set as a response variable. The relative female and male RS for each individual were calculated by dividing our estimates of the female or male RS, respectively, of each given individual by the corresponding mean RS across the sampled individuals.

We used a *gls* model to access the effects of size and flowering date on the seasonal total RS of different gender, i.e., male-phase and hermaphrodite-phase individuals. Relative total RS was set as a response variable, calculated by dividing the sum of female and male RS of an individual by the mean of the sum of female and male RS across the sampled individuals. Gender, standardized size, and standardized flowering date were set as fixed effects, along with their two-way and three-way interactions. The variance of relative total RS was allowed to differ between male-phase and hermaphrodite-phase individuals.

To test whether the earliest flowers produced by an individual had a higher probability of being entirely male, we used a generalized linear mixed model, with the probability of being a male flower set as a response variable. The flowering order of each flower from individuals producing a mixture of male and hermaphroditic flowers in Population LM was calculated according to the flowering date of the flowers. Individual identity was set as a random effect.

We used a posthoc Tukey test to test the difference in male flower probability among flowering orders.

Results

Siring ability of pollen from different sources

We assessed the self-compatibility and relative siring ability of pollen from male versus hermaphroditic flowers in Population LL1. Flowers pollinated with pollen from both the same and from different individuals all produced seeds and fruit, confirming that *P. alpina* is self-compatible. There was no difference in siring ability between the pollen from male and hermaphroditic flowers (Appendix S4). Outcrossed flowers had a significantly higher mature seed-set than selfed flowers (Appendix S4), pointing to likely inbreeding depression at the seed development stage, which we calculated as 0.15. Open-pollinated flowers had a significantly lower mature seed set compared to hand-pollinated flowers (Appendix S4), indicating a certain degree of pollen limitation.

Phase changes in the SA of individuals from one season to the next

A total of 111 individuals were marked in 2019 in Population S1, among which 22, 32, and 57 individuals were in the non-flowering phase, male-phase, and hermaphrodite-phase, respectively. All 111 individuals were still alive in 2020. A total of 13 of them (12%) changed between male-phase and hermaphrodite-phase between years (Figure 1). A total of 4 (13%) and 46 (81%), respectively, of male-phase and hermaphrodite-phase individuals, remained in the same phase. See Appendix S5 for the transition matrix of marked individuals in different phases between two years.

Phenology and mate availability at the population level

In general, we found that larger individuals were more likely to flower than smaller individuals (Appendix S6). We assessed flowering phenology and allocation in detail in Population LM in 2018. Flowering began on 29 May and ended on 29 June 2018 (149-180 on Julian day; Figure 2). Population LM comprised 625 individuals. In total, 899 flowers were produced during the season, of which 691 were phenotypically hermaphroditic and 208 were phenotypically male flowers. There were 111 individuals with only one male flower (18% of the population), 10 with multiple male flowers (1%), 342 with only one hermaphroditic flower (55%), and 162 with both male and hermaphroditic flowers (26%; Table 2). Hermaphroditic flowers (N = 103) from the subsampled individuals had a mean (± SD) of 184.8 ± 84.7 pistils and 202.3 ± 39.2 stamens, the latter being similar in number to those in male flowers (209.9 ± 38.3, $N = 41$; *t*-test: $P = 0.3$; Figure 2A and B). Such a pattern of pistil and stamen numbers is reflected in a bimodal distribution of the functional gender of the subsampled individuals (Figure 2C).

Male and hermaphroditic flowers had the same longevity but differed in their phenology, with implications for the distribution of mate availability. Specifically, both male and hermaphroditic flowers lasted on average seven days, with hermaphroditic flowers spending three days in their female stage and four days in their male stage. The flowering peak for male flowers largely coincided with the peak for the female stage of hermaphroditic flowers. Male flowers thus preceded the peak for the male stage of hermaphroditic flowers (Figure 2D). Female mate availability dropped from 0.73 to zero over the course of flowering (Figure 2E), i.e., intra-sexual competition for siring success was low at first and increased over time. The observed decline in pistil availability over the flowering season in Population LM was also found in all four of the populations sampled in the subsequent year (2019; Appendix S7).

Size- and time-dependent SA, and functional gender

The number (proportion) of subsampled individuals with only one male flower, multiple male flowers, one hermaphroditic flower, and a mixture of male and hermaphroditic flowers were 17 (0.19), 3 (0.03), 39 (0.44), and 29 (0.33), respectively (Table 1). The absolute allocation to female and male functions among individuals varied from 0 to 0.37 g and from 0.009 to 0.09 g, respectively. Absolute allocation increased with size (Figure 3A; Appendix S8), but the slope of allocation on size was greater for the female function (95% CI of the coefficient: 0.032 - 0.065) than the male function (95% CI of the coefficient: 0.006 - 0.012; interaction between sex function and size: $P < 0.01$). This pattern was confirmed for the other four populations sampled in 2019 (Appendix S6).

Absolute allocations also increased with flowering date (Figure 4A; Appendix S8), with the slope again larger for female function (95% CI of the coefficient: 0.019 - 0 .051) than for male function (95% CI of the coefficient: -0.004 - 0.002; interaction between sex function and flowering date: $P < 0.01$). Functional gender among the sampled individuals ranged from 0 to 0.75 (where 0 represents pure male and 1.0 represents pure female; see also Figure 2C), increasing with size ($P < 0.01$; Figure 3B; Appendix S9) and flowering date ($P < 0.001$; Figure

4B; Appendix S9). Small and early-flowering individuals tended to be in a male-phase, i.e., with a functional gender of zero (Appendix S10).

Size- and time-dependent prospective seasonal RS

Relative RS increased with plant size (Figure 3C; Appendix S8; $P < 0.001$), but the slope was slightly steeper for female RS (95% CI of the coefficient: 0.35 to 0.72) than for male RS (95% CI of the coefficient: 0.2 to 0.54; interaction between size and sex function: $P < 0.05$). The relationship between flowering date and RS differed between male and female functions (interaction: $P < 0.001$). Female RS increased with flowering date (Figure 4C; Appendix S8; 95% CI of the coefficient: 0.16 to 0.51), whereas male RS decreased with flowering date (95% CI of the coefficient: -0.81 to -0.48). There was a three-way interaction among size, flowering date, and sex function (Appendix S8; Appendix S11; $P < 0.05$).

Total RS increased with size (Figure 3D; Appendix S12; $P < 0.001$), but the slope was steeper for hermaphrodite-phase (95% CI of the coefficient: 0.38 to 0.73) than for male-phase individuals (95% CI of the coefficient: -0.12 to 0.29; interaction between size and gender: $P < 0.001$). Total seasonal RS decreased with flowering date for both hermaphrodite-phase and male-phase individuals (Figure 4D; Appendix S12; $P < 0.01$; interaction between flowering date and gender: $P > 0.05$).

Phenology of andromonoecious individuals

For individuals expressing andromonoecy, i.e., individuals producing both male and hermaphroditic flowers in the same season, the first flower had a highest probability of being a male flower, at a time of maximum female mate availability (Figure 5).

Discussion

We assessed the effect of plant size and the within-season timing of flowering on SA and prospective RS in the andromonoecious plant *P. alpina*. Our results corroborate theories on size-dependent SA and illustrate the likely influence of resource status on gross flowering decisions made by perennial plants over the course of their lives. In particular, they help to explain both the expression of gender diphasy in *P. alpina* as well as the evolution of andromonoecy in species with a strong temporal separation of the male and female functions in their flowers.

The mating system and inbreeding depression in P. alpina

Results from our hand-pollination treatments indicate that *P. alpina* is self-compatible and that there are no differences in the ability of pollen from male and hermaphrodite flowers of *P. alpina* to sire progeny upon pollination. Although self-incompatibility systems are common in Ranunculaceae (Allen and Hiscock, 2008), most of the species in the genus *Pulsatilla*, including *P. alpina* in this study, are self-compatible (Jonsson et al., 1991; Lindell, 1998). No difference in the siring ability of pollen from male and hermaphroditic flowers further justifies our estimate of male RS, which conforms to the general pattern found in other andromonoecious species (Solomon, 1985; Huang, 2003; Cuevas and Polito, 2004; Dai and Galloway, 2012).

The finding that artificially self-pollinated flowers of *P. alpina* produced fewer seeds than those pollinated with outcross pollen points to the expression of a degree of early-acting inbreeding depression in the species. Our estimate of inbreeding depression at the seed stage is 0.15, which should be interpreted as the low bound given that the inbreeding depression may expression at the later stages and that the inbreeding depression is in general strong in perennial plants (Angeloni et al., 2011). Nonetheless, inbreeding depression will most likely reduce the male RS estimated by our mass-action model of the individuals with multiple flowers via geitonogamy (though not for individuals with single flowers, which we assume are fully outcrossed).

Size- and resource-dependent SA

P. alpina is a perennial herb that stores its resources over winter and protects its meristems for future growth in an underground rhizome. We did not measure the size of the rhizome of the plants sampled in this study, but we suppose that above-ground plant size strongly reflects rhizome size as a function of both age and factors that impact resource gains (through photosynthesis) and resource losses (e.g., through flowering, fruiting and/or as a result of herbivory) in the previous growing season(s). The observation that small plants were less likely to flower than large plants is consistent with this supposition, as is the fact that small plants were less likely to flower in two consecutive years than were larger plants.

Our results are also consistent with the notion that flowering through the female function (in *P. alpina*, this means adopting a hermaphroditic rather than a male phase in a given season) places a heavier burden on a plant's resources than flowering just through its male function (Schlessman, 1991; Zhang et al., 2014; Bialic-Murphy et al., 2020): smaller plants were more likely to produce a single male flower, whereas larger plants produced more flowers and flowers with both male and female functions. Indeed, we found that larger individuals of *P. alpina* made a greater absolute and relative allocation to their female function than smaller plants, with a significantly steeper positive slope of absolute allocation to the female than male function. Accordingly, larger plants also expressed a more female functional gender than small plants. A similar size-dependent SA strategy has been found in other insect-pollinated perennials (reviewed in de Jong and Klinkhamer 2006) and conforms to predictions of theory on size-dependent SA in simultaneously hermaphroditic plants (Klinkhamer et al., 1997; Cadet et al., 2004).

Gender diphasy with small functional males

An important feature of the reproductive strategy of *P. alpina* is that its floral SA shows both variation on a continuum, particularly with different numbers of pistils produced in its flowers, but also discrete variation involving the production of either bisexual hermaphroditic flowers or male flowers. Significantly, male flowers do not represent just the extreme end of a male-female continuum, but rather one of two quite different modes of floral allocation, with flowers producing no pistils at all or a few hundred pistils. This means that small individuals that only produce one or (rarely) two male flowers have adopted a fully male strategy for the season, whereas larger individuals flower as hermaphrodites. To the extent that size reflects age, this dichotomy represents a strategy of gender diphasy, where individuals transition from one allocation mode to another (Schlessman, 1988). Indeed, our transition matrix reveals that about a tenth of all individuals changed their gender between consecutive seasons (see also Appendix S4).

Although gender diphasy in perennial plants most commonly involves a shift in gender expression from male to female, many species share the strategy displayed by *P. alpina* of shifting between male- and hermaphrodite-phases (Freeman et al., 1980; Schlessman, 1988), e.g., *Lilium apertum* (Zhang et al., 2014), *Lloydia oxycarpa* (Niu et al., 2017), *Panax trifolium* (Schlessman, 1991), and *Tulipa pumila* (Astuti et al., 2020). In these species, male-phase individuals are usually small and produce only one or few flowers, and it is generally thought that the small individuals in the male-phase likely contribute little to their lifetime fitness (Charlesworth, 1984; Zhang and Jiang, 2002). Indeed, studies on gender diphasic species have typically failed to show any advantages in male RS of male-phase over hermaphrodite-phase individuals in terms of pollen production, flower size, pollinator visitation rate, or pollen siring ability (Peruzzi et al., 2012; Zhang et al., 2014; Niu et al., 2017; Astuti et al., 2020). However, in most of these cases, the male flowers are smaller and produce less pollen compared to hermaphroditic flowers (Zhang et al., 2014; Niu et al., 2017; Astuti et al., 2020). This contrasts with the male flowers of *P. alpina*, which tend to produce the same number of stamens as hermaphroditic flowers. We discuss the significance of this observation in the next section.

A strategy of size-dependent SA needs to be understood in terms of both the costs and the benefits of flowering through a particular sexual function. In their model of size-dependent SA, Zhang and Jiang (2002) suggested that small individuals allocating only to male function could maximize their lifetime fitness by keeping their reproductive effort low and thereby enhancing their survival and RS in the following seasons. They argued that this should be especially so when the marginal cost of the female function is substantially higher than the male function. To some extent, this scenario would seem to apply to *P. alpina*. In a previous study (**Chapter III**; Chen and Pannell 2022), they found that individuals of *P. alpina* bear a particularly heavy cost of their female function in terms of the elongated floral stalks produced for hermaphroditic flowers but not male flowers – presumably as an adaptation for seed dispersal by wind. Small and resource-limited individuals of *P. alpina* might avoid allocation to female function and the need to produce costly floral stalks by producing male flowers partly as a strategy to enhance inter-seasonal survivorship. However, our results suggest that desisting from female allocation in *P. alpina* also has an alternative or additional explanation in terms of capitalizing on opportunities for siring success.

Implications of the timing of SA for female versus male RS

Our study stands out by having estimated not only the allocation to male and female functions of individuals of different sizes and resource status but also the time-dependent opportunities for mating and RS over the course of the flowering season. Because flowers of *P. alpina* are strongly protogynous, opportunities for mating, estimated in terms of the number of receptive pistils available per stamen, declined steeply during the course of the flowering season (see Austen et al. 2015 for a review of how the other factors may affect this relation). We estimated female RS for each plant in terms of the number of seeds it produced during the season. Prospective male RS of individuals was determined by integrating their prospective siring success in terms of a mass action model of mating over the period of their flowering, accounting for both their stamen production and the numbers of pistils for which they might compete to pollinate within successive time windows. We could thus estimate the prospective seasonal RS for each individual both as a function of its size and also in terms of when during the season it was likely to have been most successful through its male function.

Our results indicate that larger plants had greater prospective seasonal RS through both their male and female functions, but the relationship with size was significantly steeper for the female function, with an intersection between the two sex functions at mid-size. This indicates that male RS contributes relatively more to the total seasonal RS of small plants whereas, after crossing a certain size threshold, the contribution of female RS likely exceeds that of the male function – a pattern that is reflected in the overall greater female gender of larger plants. The reproductive allocation strategy of *P. alpina* thus conforms to the size-advantage hypothesis (Ghiselin, 1969; Zhang and Jiang, 2002), not only in terms of its gender diphasy, discussed above, but also in terms of the quantitative variation in gender among hermaphrodites of different sizes but flowering together in the same season.

An important implication of our results is that the dynamic nature of female mate availability over the course of the flowering season meant that plants flowering early tended to have a male-biased gender, while those flowering later had a female-biased gender. Moreover, because of the high availability of pistils to be pollinated in the early season, small plants that produce only male flowers (see above) had high prospective siring success despite their small size, i.e., their male-only allocation is probably not just an outcome of resource constraints facing small individuals, as often supposed for other species (Zhang and Jiang, 2002; Peruzzi et al., 2012), but also an adaptation to capitalize on mating opportunities. Indeed, our results indicate that although small male-phase individuals obviously gained no fitness via their female function, the prospective total seasonal RS of some male-phase individuals is likely to have been higher than that of some hermaphrodite-phase individuals of similar size.

Most studies have not considered the effect of both plant size and flowering phenology (and patterns of mate availability) jointly (but see Schlessman et al. 1996; Schlessman and Graceffa 2015). Consequently, the potentially high contribution to lifetime fitness of plants via their male function when small, and thus the functional significance of small males in gender diphasic species (Kudo and Maeda, 1998; Zhang and Jiang, 2002), may hitherto have been under-appreciated. According to our reasoning, we should expect male-phase individuals in diphasic populations to be early flowering in protogynous species, as in *P. alpina*, and late flowering in protandrous species. Indeed, in protandrous dwarf ginseng (*Panax trifolium*), male-phase individuals were found to flower later in the season compared to hermaphrodite-phase individuals and were highly synchronized with the phenology of the female-stage of hermaphroditic individuals (Schlessman et al., 1996). These patterns are generally consistent with ideas from SA theory and the dynamic nature of the mating environment in dichogamous species (Brunet and Charlesworth, 1995; Austen and Weis, 2014) and are coherent with other empirical studies on protogynous species (Huang et al., 2004; Guitián, 2006).

Andromonoecy as the resolution of intersexual conflict due to dichogamy

Our analysis of SA and components of prospective RS for individuals of *P. alpina* in the context of their flowering phenology also points to an explanation for the evolution of andromonoecy in dichogamous species. Andromonoecy is a heteromorphism involving the production of male and hermaphrodite flowers by the same individual. Although small individuals of *P. alpina* produce only male flowers, over the course of their lives all individuals likely produce both male and hermaphroditic flowers, so the sexual system can be considered andromonoecious. Our results suggest that the male flowers of large individuals likely promote male RS in the same way as the early flowering of small male-phase individuals, notably by resolving intersexual conflict faced by individuals with bisexual flowers.

As our analysis of phenology clearly shows, plants stand to achieve substantial siring success by dispersing pollen early in the flowering season. But because *P. alpina* is strongly protogynous, individuals producing bisexual flowers must delay the onset of their male function until they have passed through the female stage. This immediately suggests that andromonoecy in *P. alpina* might be interpreted as a strategy to advance the timing of the male function of individuals by suppressing their female function in some flowers. The resolution of the sexual conflict within flowers through the production of all-male flowers rather than via a modification of the dichogamy responsible for the conflict is consistent with the fact that species in the genus *Pulsatilla* are all protogynous and that patterns of dichogamy tend to be much more phylogenetically conserved than phenology and could thus be viewed as a phylogenetic constraint (Lloyd and Webb, 1986; Jonsson et al., 1991; Routley et al., 2004).

The function of male flowers in andromonoecious species has attracted substantial speculation (Tomaszewski et al., 2018), but empirical evidence for the various ideas put forward remains ambiguous. For example, male flowers in *Passiflora incarnata* sired on average twice as many seeds as hermaphroditic flowers, largely as a result of greater pollen production and less self-pollen deposition (Dai and Galloway, 2012), and male RS in *Solanum carolinense* increased with the proportion of male flowers but not with the total number of flowers, likely because the absence of pistils in male flowers allowed bumblebees to remove pollen more efficiently (Elle and Meagher, 2000). In contrast, Podolsky (1993) found that hermaphroditic flowers in *Besleria triflora* dispersed substantially more pollen than male flowers over an average flower's lifetime, implying that male flowers contributed relatively little directly to male RS. Similarly, male flowers in *Anticlea occidentalis* promoted female mating quality in terms of outcrossing rate and mate diversity but did not affect male RS in terms of seed sired (Tomaszewski et al., 2018).

Our study illustrates the insights that can be gained by studying the life history and phenology of plants jointly rather than independently. Moreover, by calling attention to the potentially high contribution to seasonal male RS made by male flowers produced when mate availability is particularly high, and by drawing a link with gender diphasy, our study exposes a simple but largely overlooked explanation for the evolution of andromonoecy (Pellmyr, 1987; Schlessman, 2010). Indeed, both gender diphasy and andromonoecy have been found in many dichogamous species in Ranunculaceae (Pellmyr, 1987; Lindh, 2017), Liliales (Peruzzi, 2012), and Apiales (Schlessman, 2010), and Schlessmann (2010) has argued that the flowering order of male and hermaphroditic flowers in such species should depend on whether they are protandrous or protogynous. In common with *P. alpina*, these species are also often perennial herbs with underground storage organs, a relatively short flowering season, and a relatively high cost of female function associated with either the dispersal of seeds by wind from costly inflorescence stalks or by animals attracted to costly fleshy fruits.

Taken together, our results show that resource status affects the absolute and relative resource allocation to female function in *P. alpina*, while the timing of flowering likely determines RS through its male function. This is consistent with the general view that female RS is commonly limited by resources, whereas male RS is limited by mate availability (Bateman, 1948; Charnov, 1979). The gender-diphasic and andromonoecious strategy displayed by *P. alpina* means that individuals can adjust their SA in response to resource status and mate availability at both the individual level, from male-phase to hermaphrodite-phase individuals, and the flower level, from phenotypically male flowers to hermaphroditic flowers – a dichotomy not predicted by theory for SA that consider size and the timing of flowering independently (Brunet and

Charlesworth, 1995; Klinkhamer et al., 1997). As a consequence, individuals may avoid investing in their female function when resources are limited, consistent with predictions for the evolution of andromonoecy (Spalik, 1991; de Jong et al., 2008), and by producing male flowers early in the season when mate availability is high.

Acknowledgments

This study was funded by a grant from the University of Lausanne. We thank the Canton of Vaud and the Communes of Bex and Ormont-Dessous for access to field sites; Nora Khelidj for help with fieldwork; and members of the Pannell lab for valuable discussions.

Table 1. Composition and number of flowers of individuals flowering as only male or as a hermaphrodite in Population LM. Shown are the number and proportion of individuals in each given phase, as well as the number and proportion that were subsampled for measurements. Note that the phenotypic gender of individuals producing only male flowers was classified as male (M-phase individuals) while that of individuals producing only hermaphrodite or a mixture of male and hermaphrodite flowers was classified as hermaphrodite (H-phase individuals).

	N	Proportion	Subsampled	Proportion
1-flowered M-phase	111	.18	17	.19
1-flowered H-phase	342	.55	39	.44
2-flowered M-phase	8	.01	3	.03
2-flowered H-phase	104	.17	17	.19
>2-flowered M-phase	2	.003	0	0
>2-flowered H-phase	58	.09	12	.14
Total	625	1	88	1

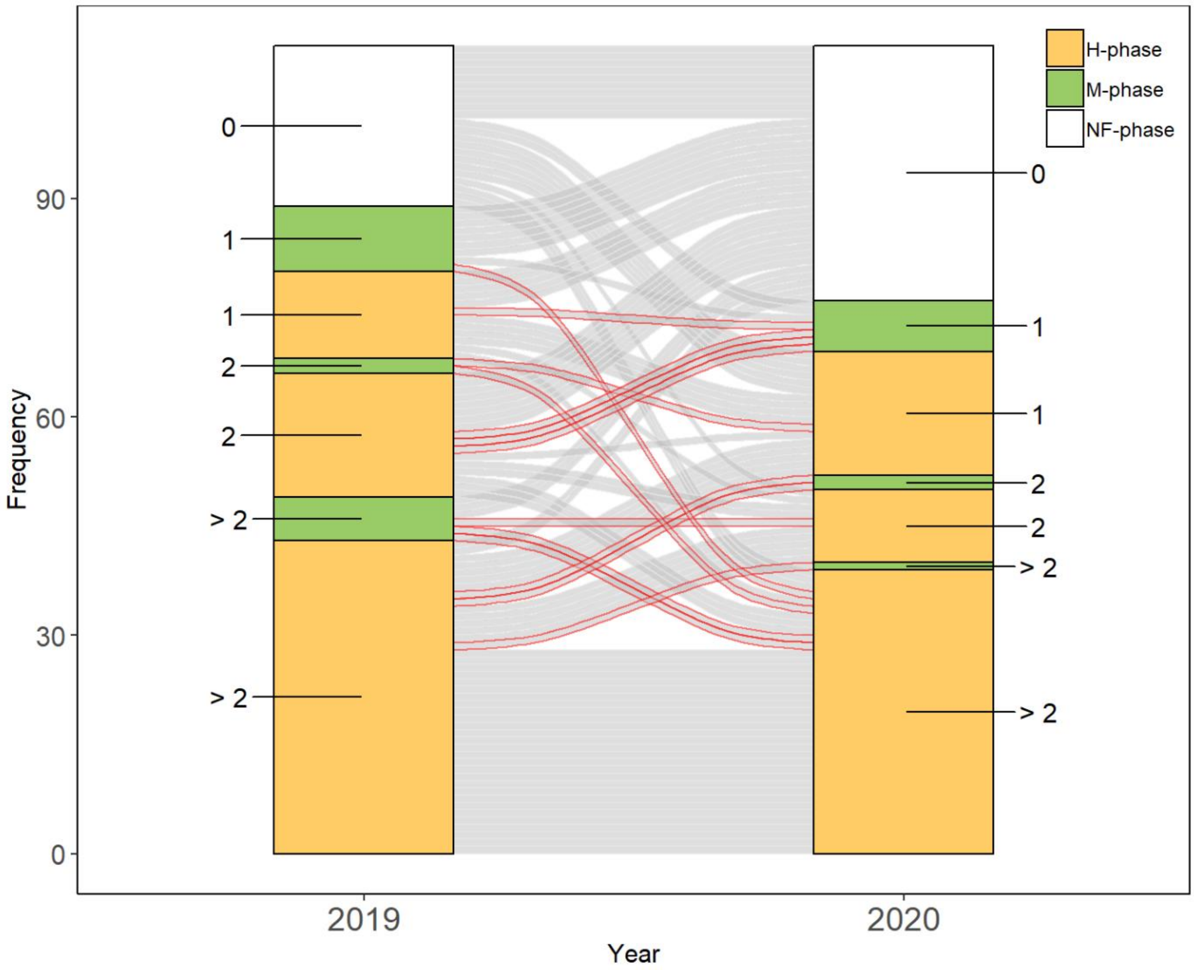


Figure 1. Flow diagram showing the changes of gender phase in marked individuals over two years in Population S1 (N = 111). The red lines represent changes in gender between an M-phase and an H-phase (N= 13, 12%). Individuals were separated into seven gender classes according to their gender phase (non-flowering, NF-phase; male, M-phase; hermaphrodite, H-phase) and the number of flowers (non-flowering, one-flowered, two-flowered, and more-than-two-flowered).

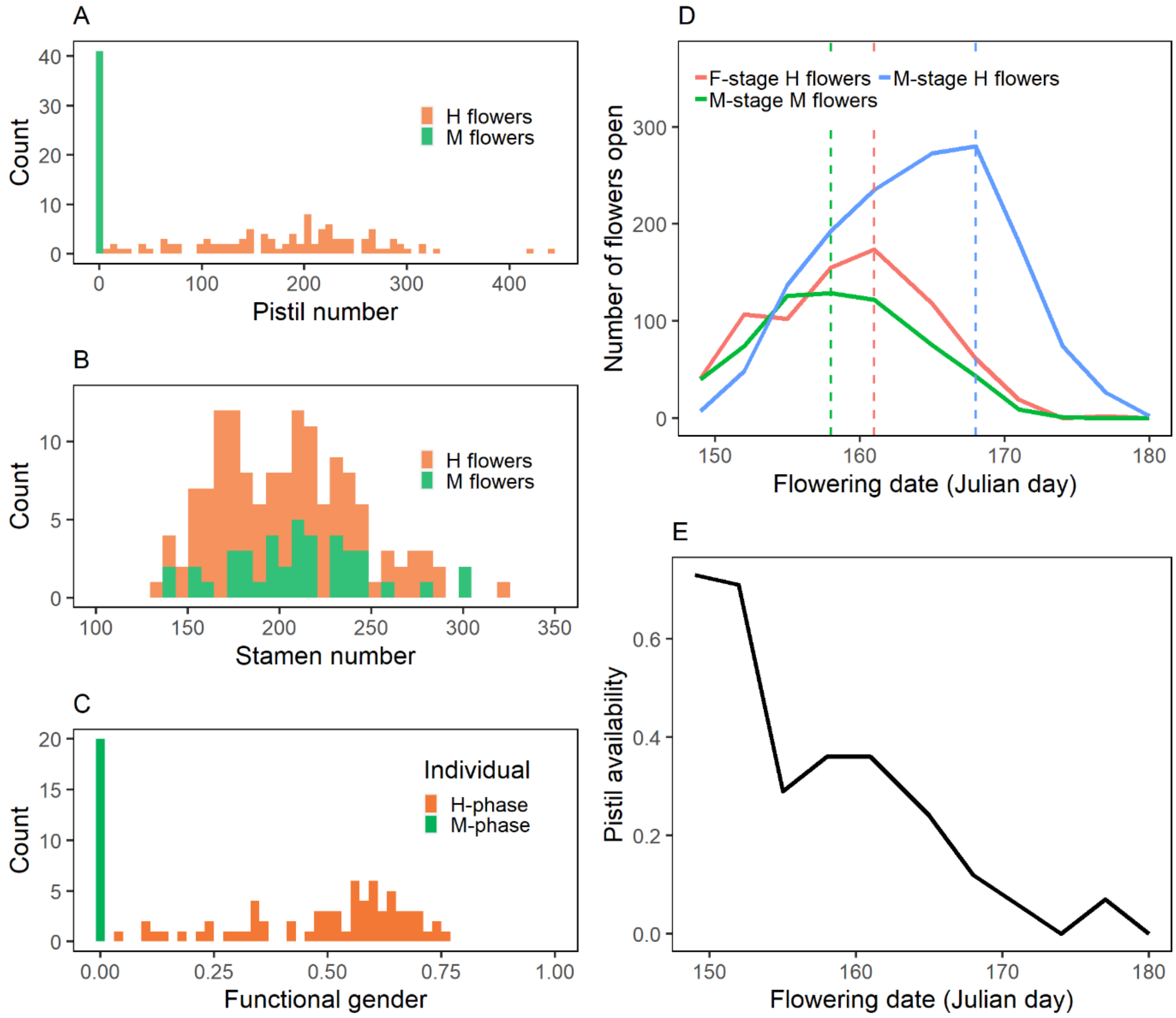


Figure 2. Histograms of the pistil number (A) stamen number (B) and functional gender (C) of sampled flowers, number of flowers at male and female stages (D), and pistil availability (E) across the flowering season in Population LM. ‘M flowers’ and ‘F flowers’ refer to phenotypically male and hermaphroditic flowers, respectively. ‘M-phase’ and ‘H-phase’ individuals refer to individuals flowering with only male or with hermaphrodite (and potentially male) flowers. ‘F-stage’ and ‘M-stage refer to flowers in their female or male stages (along their protogynous progression). (B) and (D) Phenotypically hermaphroditic (hermaphrodite flowers, *N* = 103) and male (Male flowers, *N* = 41) flowers are colored orange and green, respectively. The distributions of the pistil number (A) and functional gender (C) are bimodal whereas that of the stamen number (B) is unimodal. (D) Male flowers have only a male stage (M-stage M flowers, green line) while hermaphroditic flowers are first in their female stage (F-stage H flowers, orange line) and then in their male stage (M-stage H flowers, blue line). Note that the flowering peak for male flowers (green dashed line) is earlier than that for male-stage hermaphroditic flowers (blue dashed line). (E) Pistil availability, which reflects the ratio of

available pistils to stamens at the population level, drops toward zero at the end of the flowering season.

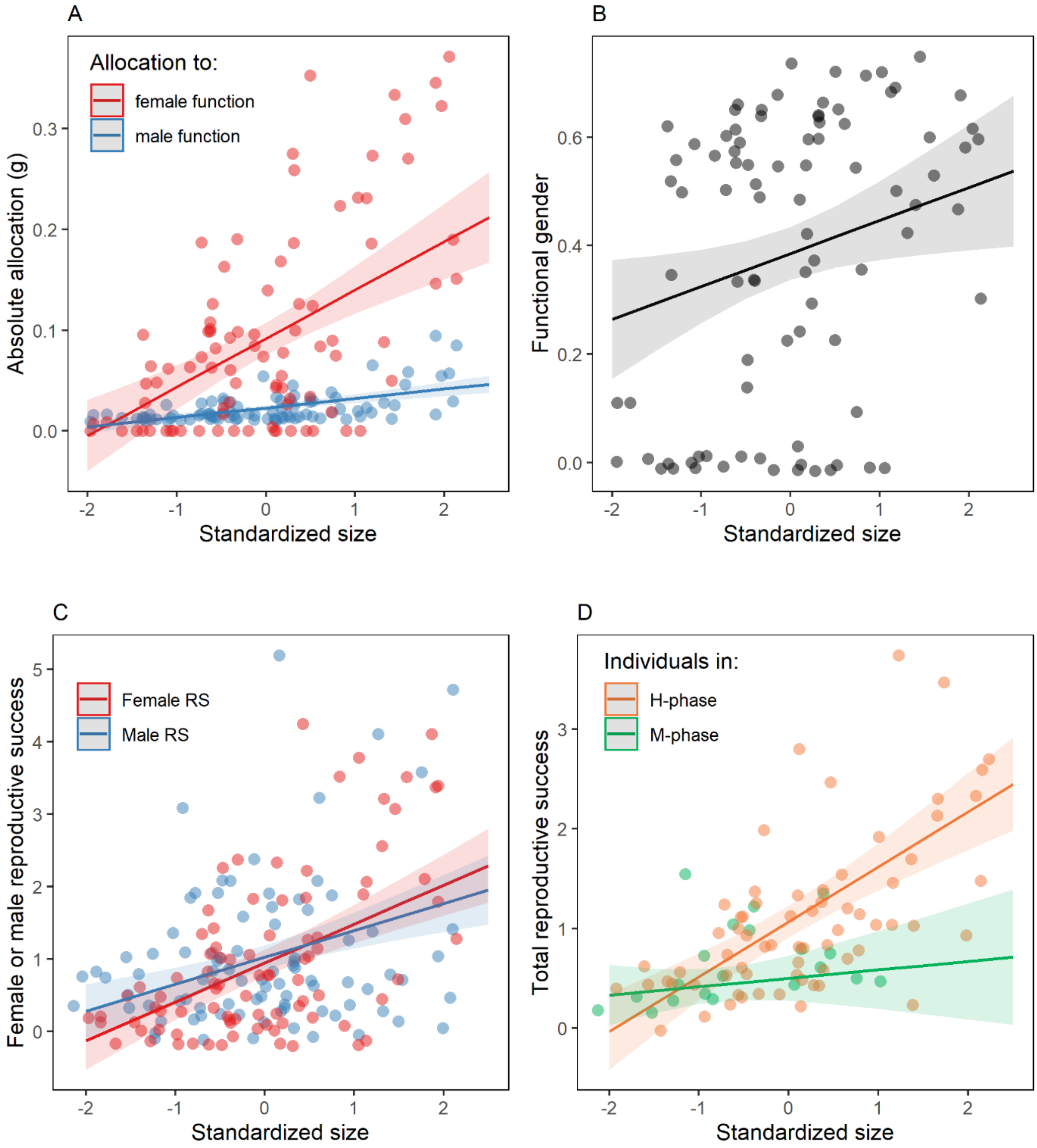


Figure 3. The effect of plant size on: (A) sex allocation to female and male functions (both slopes > 0, *P* < 0.001; female slope > male slope, size x sex interaction *P* < 0.001); (B) functional gender, calculated as femaleness (slope > 0, *P* < 0.01); (C) RS for individuals via their male and female functions (both slopes > 0, *P* < 0.001; female slope > male slope, size x sex interaction *P* < 0.05); and (D) total RS for male-phase and hermaphrodite-phase individuals (both slopes > 0, *P* < 0.001; H-phase slope > M-phase slope, size x gender interaction *P* < 0.001), as predicted by models fitted to data from Population LM. See text for details. 'M-phase' and 'H-phase' individuals refer to individuals flowering with only male or with

hermaphrodite (and potentially male) flowers. Each individual contributes two points representing its female and male allocation in (A) and its relative female and male RS in (C). The 95 % confidence interval of the estimates is shown around the regression lines.

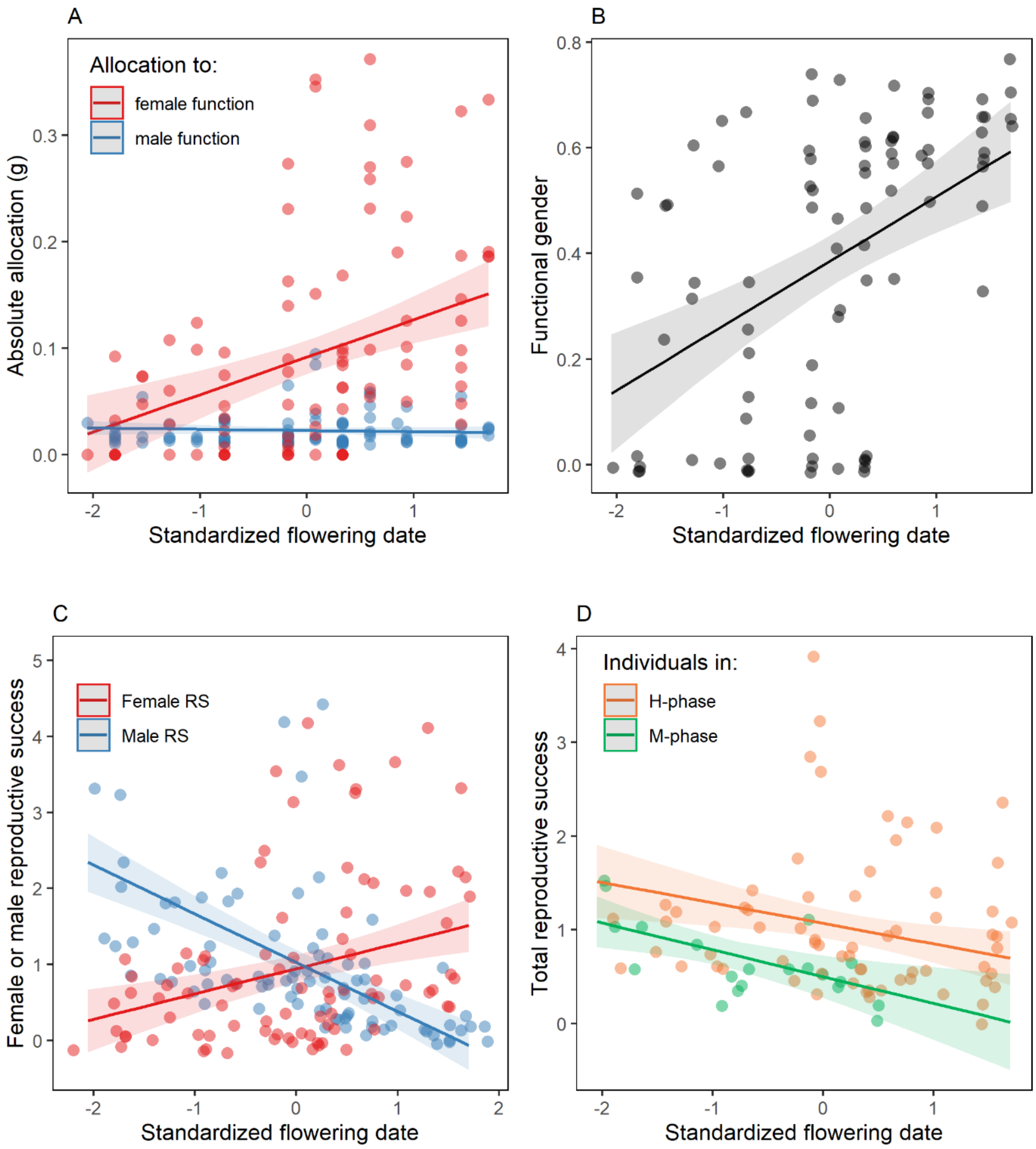


Figure 4. The effect of mid flowering date on: (A) sex allocation to female and male functions (female slope > 0, $P < 0.001$; flowering date x sex interaction $P < 0.001$); (B) functional gender, calculated as femaleness (slope > 0, $P < 0.001$); (C) RS for individuals via their male and female functions (female slope > 0, male slope < 0; flowering date x sex interaction $P < 0.001$); and (D) total RS for Male-phase and Hermaphrodite-phase individuals (both slopes < 0, $P < 0.01$; flowering date x gender interaction $P > 0.05$), as predicted by models fitted to data from Population LM. Each individual contributes two points representing its female and male allocation in (A) and its relative female and male RS in (C). ‘M-phase’ and ‘H-phase’

individuals refer to individuals flowering with only male or with hermaphrodite (and potentially male) flowers. The 95 % confidence interval of the estimates is shown around the regression lines.

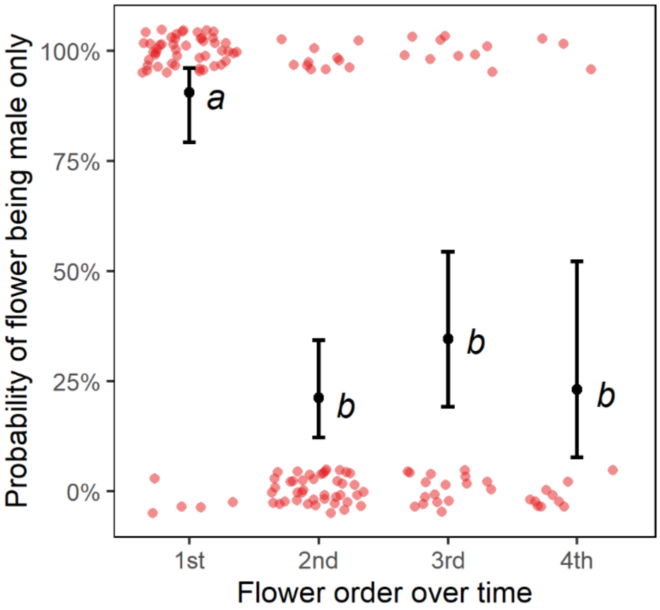


Figure 5. Probability that individual flowers are male only (have stamens but not pistils) as a function of their opening order on an individual. The first flowers to open on multi-flowered individuals have a much higher probability of being male than flowers opening subsequently. Data are from Population LM and are based on 144 flowers over 54 individuals. The probabilities are shown with the 95 % confidence interval predicted by the model (see text for details). Letters indicate means that are not significantly different from one another, based on a Tukey test (*P* > 0.05).

Supplementary Materials

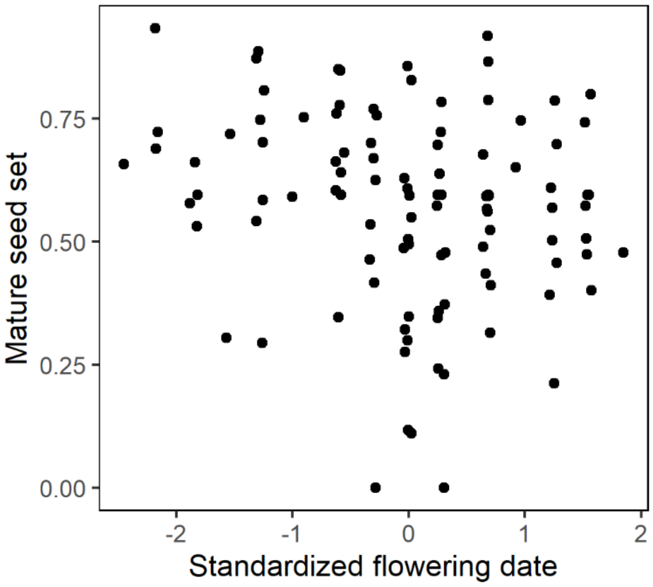
Appendix S1. Descriptions of the study populations and the studies conducted in them from 2018 to 2020.

Population	Location	GPS position	Altitude (a.s.l.)	*N*	Flower season^{+}	Habitat	Note	SA and RS estimates (2018)	Reproductive biology (2018)	Generality of SA (2019)	Gender change (2019-2020)
LM	Les Mosses	46°23′57″N 7°04′52″E	1694	About 600	E	enclosed grassland	plant size mostly small	O			
LL1	Lac Lioson	46°23′08″N 7°07′23″E	1951	> 1,000	E	open grassland	steep slope		O	O	
LL4	Lac Lioson	46°22′57″N 7°07′11″E	1983	> 1,000	L	open grassland				O	
S1	Solalex	46°17′36N 7°09′11″E	1723	> 1,000	E	open grassland	population fenced			O	O
S2	Solalex	46°16′37″N 7°09′32″E	2122	> 1,000	L	open grassland	steep slope			O	

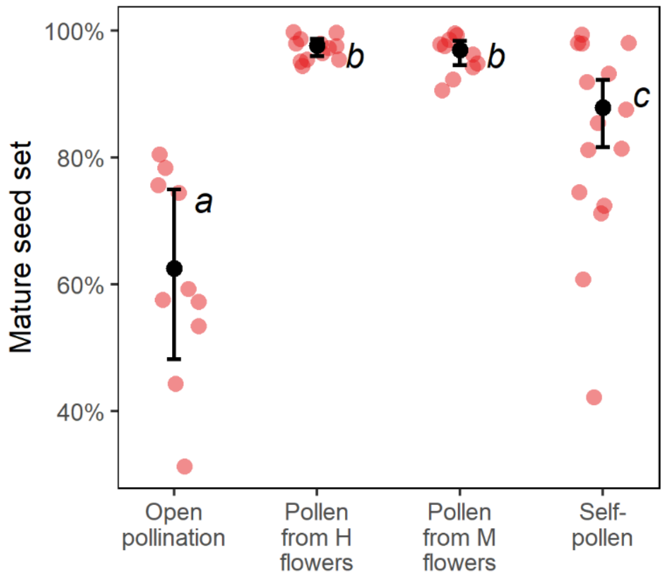
+ Flowering season indicates whether the population starts flowering in late May to early June (E) or late June to July (L).

Appendix S2. Description of phenotypic classes at different levels used in this study.

Levels	Phenotypic classes	Description
Individual-level	Male-phase individual	Individuals with male function only, i.e., only male flowers.
	Hermaphrodite-phase individual	Individuals with both male and female functions, i.e., with hermaphroditic and potentially male flowers.
Flower-level	Male flower	Flowers with stamens only
	Hermaphroditic flower	Flowers with both stamens and pistils
Intra-flower level	Female-stage flower	Hermaphrodite flowers with receptive pistils (the Female stage precedes the Male stage in hermaphrodite flowers).
	Male-stage flower	Male or hermaphrodite flowers with dehiscent stamens.



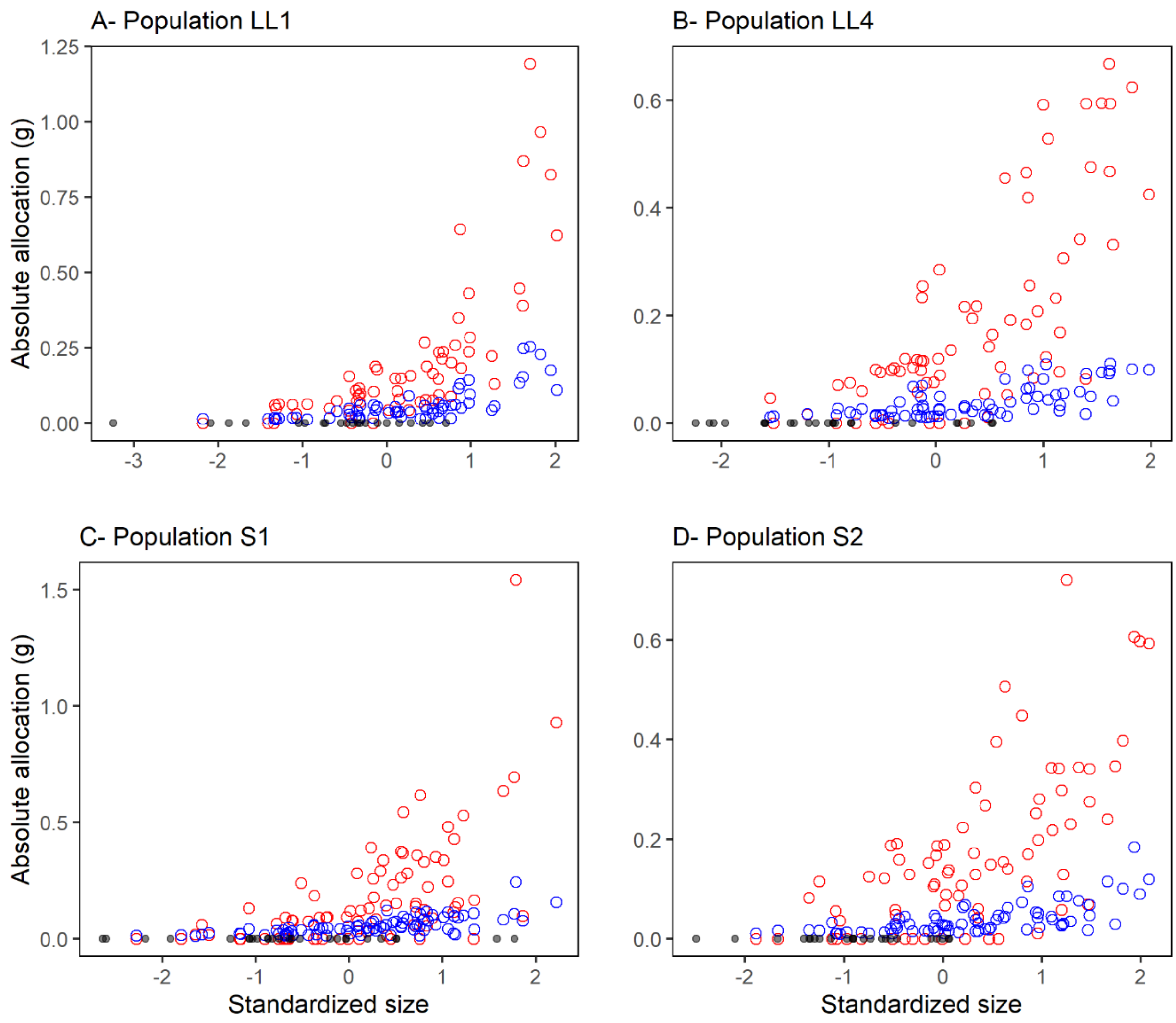
Appendix S3. Mature seed set per flower as a function of the standardized flowering date in Population LM in 2018. The mature seed set of each flower is independent of the flowering date ($P > 0.05$). Each point represents one hermaphroditic flower ($N = 103$ flowers from 68 individuals). The results indicate that there is likely no change in the degree of pollen- and resource-limitation across the flowering season regarding reproduction via the female function.



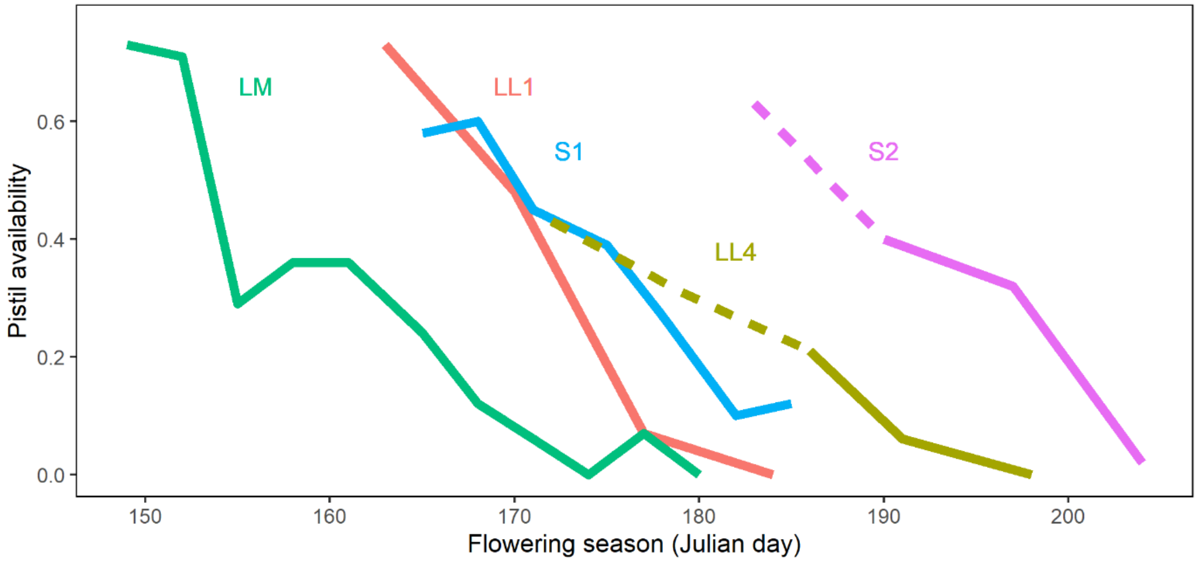
Appendix S4. The effect of artificial pollination treatment on mature seed set in Population LL1. Pollination treatments included open pollination (*N* = 10), artificial outcrossing with pollen from hermaphrodite flowers (*N* = 12), outcrossing with pollen from male flowers (*N*= 10), and artificial selfing (*N* = 15). The mature seed sets are shown with the 95 % confidence interval predicted by a generalized linear mixed model. Letters indicate means that are not significantly different from one another, based on a Tukey test (*P* > 0.05).

Appendix S5. Transition matrix of marked individuals at different flowering states or phases between two years in population S1 (*N* = 111). The rates in bold indicate a transition between male-phase (M-phase) and hermaphrodite-phase (H-phase) individuals.

		2020							
		Non-flowering	1-flowered M-phase	1-flowered H-phase	2- flowered M-phase	2-flowered H-phase	> 2-flowered M-phase	> 2-flowered H-phase	*N*
	Non-flowering	.45	.09	.27	.05	0	0	.14	22
	1-flowered M-phase	.78	.11	0	0	0	0	**.11**	9
	1-flowered H-phase	.42	**.08**	.33	0	.08	0	.08	12
2019	2- flowered M-phase	.47	.18	**.06**	0	**.12**	0	**.18**	17
	2-flowered H-phase	0	0	.5	0	0	0	.5	2
	> 2-flowered M-phase	.5	0	0	0	**.17**	0	**.33**	6
	> 2-flowered H-phase	.05	0	.12	**.05**	.12	**.02**	.65	43
	N	35	7	17	3	9	1	39	



Appendix S6. Plant size and absolute allocation to male and female functions in Populations LL1 (A), LL4 (B), S1 (C), and S2 (D). Each flowering plant is represented by two circles for its female (red circle) and male functions (blue circle). Non-flowering individuals are represented by grey points. The sample size of flowering and non-flowering individuals in Populations LL1, LL4, S1, and S2 are 61, 70, 82, and 75 and 21, 24, 26, and 24, respectively.



Appendix S7. Changes in pistil availability across the flowering season in five populations. Pistil availability was monitored in the spring of 2018 (LM) and 2019 (LL1, LL4, S1, and S2). The observations in Populations LL4 and S2 were conducted from the peak of the flowering season rather than from the early flowering season; the dashed lines are thus extrapolations to the inferred starting of the flowering date based on the assumption of a linear decrease in pistil availability over the flowering season from the field data.

Appendix S8. Summary table of the effects of plant size, flowering date, and sex function (male or female) on absolute sex allocation (SA) and reproductive success (RS). Absolute allocation was calculated as the dry mass of pistils (female function) and stamens (male function) of each individual. RS was estimated by the number of mature seeds and by a mass-function model for female and male functions respectively (see main text for details).

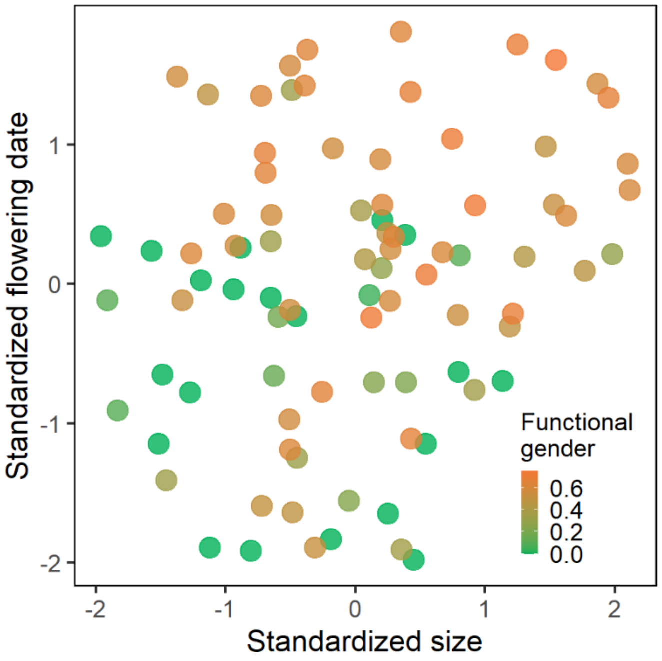
	Absolute allocation (*gls*)+			Relative RS (*gls*) +		
	df	LRT	*P*	df	LRT	*P*
Size	1	4.77	< .001	1	40.8	< .001
Flowering date	1	16	< .05	1	6.42	< .05
Sex function	1	43.89	< .001	1	0	n.s.
Sex function x Size	1	29.6	< .001	1	4.44	< .05
Sex function x Flowering date	1	20.02	< .001	1	46.32	< .001
Size x Flowering date	1	.59	n.s.	1	.62	n.s
Sex function x Size x Flowering date	1	4.6	< .05	1	6.58	< .05

Note—Individual identity was set in a compound symmetry structure in the two models to take into account the correlation of male and female sex function from the same individual.

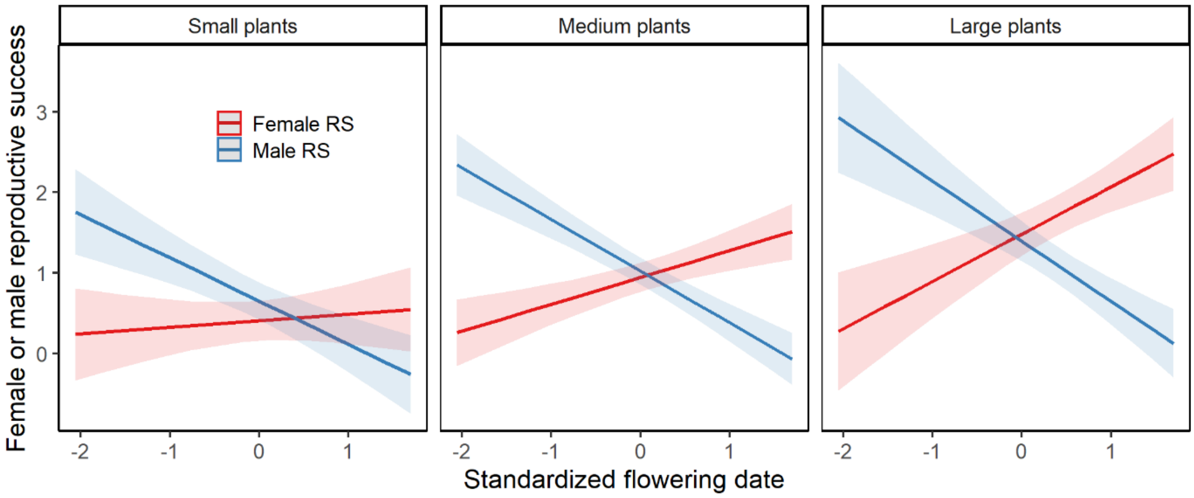
+ Variance was allowed to vary between sex functions in the *gls* models (see main text for details).

Appendix S9. Summary table of the effects of size and flowering date on functional gender. Functional gender was calculated in terms of femaleness (see main text for details).

	Functional gender (*lm*)		
	df	Sum of sq	*P*
Size	1	.34	< .01
Flowering date	1	1.22	< .001
Size x Flowering date	1	.01	n.s.



Appendix S10. Effects of plant size and flowering date on functional gender of sampled individuals in Population LM. Each point represents one individual and the color of the point indicates its functional gender calculated in femaleness.



Appendix S11. Plots showing the three-way interaction between size, flowering date, and sexual function on components of reproductive success (RS) in Population LM, as predicted by the model (see main text for details). Early flowering favors male RS for all plant sizes. However, female RS increases less steeply with flowering date in small plants (see Appendix S8; interaction among size, flowering date, and sex function: $P < 0.05$). The 95 % confidence interval of the estimates is shown around the regression lines.

Appendix S12. Summary table of the effects of size, flowering date, and phenotypic gender (male phase or hermaphrodite phase) of individuals on relative total reproductive success. The total RS of the individuals was calculated as the sum of female and male RS (see main text for details).

	Relative total reproductive success (*gls*)[+]		
	df	LRT	*P*
Size	1	24.79	< .001
Flowering date	1	8.96	< .01
Phenotypic gender	1	6.28	< .05
Phenotypic gender x Size	1	13.07	< .001
Phenotypic gender x Flowering date	1	.756	n.s
Size x Flowering date	1	.02	n.s.
Phenotypic gender x Size x Flowering date	1	.57	n.s.

Note—+ Variance was allowed to vary between phenotypic genders in the *gls* model (see main text for details).

CHAPTER II:

A fitness landscape perspective on sex-allocation theory helps to explain the complexities of male and female reproduction in perennial hermaphroditic plants

Kai-Hsiu Chen and John R. Pannell

(in preparation for *Evolution*)

Abstract

The dependency of reproductive success on sex allocation to female and male functions determines the optimal sex allocation strategy of a hermaphroditic organism. In keeping with the fundamental framework of sex allocation theory based on the idea of fitness ‘gain curves’, most empirical estimates of this dependency have viewed sex allocation as a single variable that captures both male and female allocation on a tradeoff continuum from male to female. In this study, we adopt a new approach that maps components of reproductive success on a fitness landscape defined by allocation to male and female functions independently. This approach allows for the interesting and important possibility that allocation to one sexual function might have direct fitness consequences for the other, and vice versa, so that the effects of male and female allocation go beyond a simple allocation tradeoff. We used microsatellite markers to estimate fitness components for individuals in a population of the insect-pollinated perennial herb, *Pulsatilla alpina*, which displays an enormous natural variation in its sex allocation. *P. alpina* has a mixed-mating system with strong inbreeding depression, and that outcross pollen is dispersed over short distances. The intra-floral selfing rate depended positively and negatively on the male and female allocation, respectively, leading to an accelerating female gain curve and a negative dependency of female reproductive success on male allocation due to seed discounting. Our analysis further points to a rugged landscape of fitness peaks and valleys and an interaction between sex allocation to the two sex functions on total reproductive success. Moving from a perspective of fitness gain curves to a fitness surface provides a novel explanation for the evolution of sex allocation involving andromonoecy and gender diphasy in *P. alpina* and other similar perennial herbs. It also illustrates a potentially profitable way of advancing our understanding of sex allocation studies on reproductive strategies in plants more generally.

***Keywords*:** Andromonoecy; Sexual system; Male fitness; Fitness surface; Sexual interference

Introduction

The sex allocation of flowers and individuals is the result of responses to natural selection on allocation of limited resources to female and/or male components of reproduction. The theory of sex allocation provides a simple and powerful framework for interpreting reproductive strategies in terms of how allocation to each sexual function translates into reproductive success. Classical models assume a linear trade-off in the allocation to the two sex functions within a fixed amount of resources and typically depict the gain curves with a fixed exponential function independent of the amount of resources to the opposite sex. Under these assumptions, a sex-allocation strategy is interpreted as stable when the marginal gains of reproductive success through the two sex functions are equal and the total reproductive success is maximized (Charnov, 1979; Charlesworth and Charlesworth, 1981; Klinkhamer et al., 1997). This modeling framework has been useful in providing a guide as to when we might expect populations, for instance, to be hermaphroditic, dioecious, gynodioecious, or androdioecious (Charlesworth and Charlesworth, 1978, 1981; Charnov, 1982; Charlesworth, 1984). However, the framework has been criticized because it is so challenging to test empirically.

Empirical tests of sex allocation theory are challenging because they ultimately require estimates of fitness components for male and female functions involving the tracking of progeny fates (Charlesworth and Morgan, 1991; Campbell, 2000). Predictions have been borne out in qualitative terms by comparisons among species or populations that differ in their patterns of inbreeding or dispersal (Goldman and Willson, 1986; Charnov, 1987), but we are only beginning to document the functional relationship between allocation to a particular sex and fitness gained through that sex. For example, studies have attempted to quantify the dependency of male reproductive success on variables reflecting male allocation, e.g., flower number or biomass of male organs, using paternity analysis with genetic markers in species with different pollination systems, e.g., bumble-bee pollinated *Solanum carolinense* (Elle and Meagher, 2000), humming-bird pollinated *Ipomopsis aggregata* (Campbell, 1998), or wind-pollinated *Ambrosia artemisiifolia* (Aljiboury and Friedman, 2022).

The foundational theory is formulated in terms of a map of fitness components to sex allocation along a single axis, specifically in terms of the fraction of total reproductive resources allocated to one sex (Charnov, 1979, 1982; Charlesworth and Charlesworth, 1981; Charlesworth and

Morgan, 1991). Commonly, this map is construed in terms of fitness gain curves whose shape allows us to predict a populations sexual system: hermaphroditism should be favored when gains in fitness saturate with increasing allocation to one sex or the other, whereas dioecy should be favored when the fitness gain curves are accelerating. Various ecological factors that may govern the shape of the dependency have been the subject of much verbal discussion though empirical evidence remains scarce (Charnov, 1982; Campbell, 2000). For instance, it is generally thought that the male fitness gain curve will tend to be a saturating function of male allocation in animal-pollinated plants (due to local mate competition or as a result of the pollen grooming behavior of insect pollinators; Lloyd, 1984). In contrast, the male fitness gain curve under wind-pollination is expected to be a linear function of allocation (Brunet, 1992). Moreover, female gain curves are generally thought to be linear as allocating more resources to the female function leads to produce more seeds whereas local resource competition among related seedlings, e.g., as a result of passive seed dispersal, is likely to lead to a saturating female gain curve (Lloyd, 1984a).

While the mapping of fitness components to the one-dimensional variable of sex allocation has been heuristically useful, in reality, the effects of flowering and mating strategies on plant fitness components are multidimensional and potentially interactive. For example, sex allocation to one sex may have implications for reproductive success not only through that sexual function but also through the other, but this possibility has been largely ignored in studies of sex allocation. Thus, the reproductive success of female function is likely to be affected by the sex allocation to male function not only simply as a result of allocation trade-off but also as a function of its effects on self-fertilization, especially in self-compatible hermaphrodites with a mixed mating system (Lloyd, 1982). If the selfing rate depends positively on the sex allocation to male function and the inbreeding depression is not zero, female reproductive success may decline with increased male sex allocation not only because of a sex-allocation tradeoff, but also because of the effects of seed or ovule discounting (*sensu* Lloyd 1992), i.e., the reduced production of outcrossed seeds with high fitness. In this situation, seed discounting seems likely to cause the female gain curve to accelerate, because female reproductive success will be least compromised by selfing when most of the reproductive resources are allocated to the female function and allocation to the male function in flowers is minimized (de Jong et al., 1999). Although seed or ovule discounting has been demonstrated in some species in which the selfing rate increases with total flower number as a result of geitonogamy (Snow et al., 1996; Vrieling et al., 1999; Eckert, 2000; Karron et al., 2003), our understanding of how the shape of the female gain curve should be affected by male sex allocation remains poor, not least because studies of the effects of sex allocation on fitness have tended to ignore the effects of potential selfing and inbreeding depression.

Just as female reproductive success may depend on male allocation, so too male reproductive success may depend on female sex allocation in ways that go beyond a simple tradeoff. For instance, an increase in female sex allocation may lead to a decrease not only in the amount of pollen produced, but also in the successful dispersal of pollen as a result of physical interference of female and male sexual organs as they interact with pollinators in hermaphroditic flowers (Barrett, 2002). In andromonoecious *Solanum carolinense*, for example, which produces male and hermaphroditic flowers that differ qualitatively in their female sex allocation, it has been suggested that female sex allocation in hermaphroditic flowers interferes with pollen removal by bumble bees, reducing their male reproductive success, a problem obviated in flowers with only male function (Elle and Meagher, 2000). While it seems clear that the sexual strategies of flowers may often affect both the male and female components of reproductive success in ways that go beyond simple allocation tradeoffs, it remains largely unclear how reproductive success through each sexual function depends on allocation to the opposite sex quantitatively.

In the present study, we attempt to understand the variation in sex allocation among flowers and individuals of a perennial herb by extending a depiction of gains in reproductive success in terms of sex allocation from one dimension to two dimensions. Our perspective thus combines a fitness landscape perspective (*sensu* Wright 1982) with key ideas in sex-allocation theory to interpret floral variation in allocation to male and female functions. Our approach will ultimately be to depict fitness as troughs and peaks on a surface defined by male allocation on one axis and female allocation on the other – rather than as a function of the single variable ‘sex allocation’ that combines both components of allocation as a single variable simply by assuming a 1:1 tradeoff. The landscape we envisage fully accommodates the possibility of a sex-allocation tradeoff, but it also allows for the possibility that other factors linked to male and/or female allocation could have direct effects on one or both components of reproductive success. The case we consider illustrates how the selfing rate affects female reproductive success that depends on male allocation. Although mapping reproductive success to genotypes with continuous traits in a fitness landscape has been common in evolutionary biology (Bataillon et al., 2022), including a number of recent studies (e.g., Nakahara et al. 2018; Tonnabel et al. 2019; Aljiboury and Friedman 2022), to my knowledge our approach has never been applied in empirical studies of sex allocation.

Our study specifically asks how floral sex allocation to both female and male functions determines female, male, and total reproductive success in the andromonoecious and self-compatible perennial herb *Pulsatilla alpina* (Ranunculaceae). *P. alpina* shows wide natural variation in pistil and stamen numbers within flowers and size-dependent sex allocation at the plant level: whereas larger individuals allocate absolutely and relatively more resources to their female function in terms of pistil number by producing a mixture of phenotypically male and hermaphroditic flowers (Chapter I; Chen and Pannell, 2023), small individuals usually produce only one male or one hermaphroditic flower. The species thus provides an exceptional opportunity to explicitly assign reproductive success, especially the male reproductive success estimated by genetic markers, to sex-allocation phenotypes. Our study involved estimating the mating system and measures of male and female reproductive success of *P. alpina* to determine the shape of fitness gain curves and gain surfaces. We selected a population predominantly comprised of single-flowered individuals because this allowed us to link estimates of male fitness to the specific sexual phenotype of individual flowers. We were particularly interested in understanding the evolution of both andromonoecy and size-dependent sex allocation in *P. alpina*.

We used microsatellite markers to estimate selfing rates, paternity, and pollen dispersal distances, and we inferred the level of inbreeding depression in the population by comparing inbreeding coefficients between seed and adult individuals (Ritland, 1990). We then asked how floral sex allocation to female and male functions affects female, male, and total reproductive success using three complementary regression approaches. First, we used regression with a power function to characterize univariate fitness gain curves in terms of the total effects of sex allocation on reproductive success via female and male functions. Second, we conducted selection gradient analyses using multivariate regression to determine the extent to which reproductive success depends on sex allocation to both sexes independently, thus allowing us to infer potential synergy or interference between the sexes in how allocation affects fitness.

Finally, we used nonparametric regression to determine the inter-dependency and non-linear relationship between reproductive success and sex allocation to the two sex functions on a two-dimensional fitness landscape. To understand how inbreeding depression suffered by selfed offspring modifies the shape of the gain curves or the fitness landscapes, we compared our inferences between scenarios using our estimate of inbreeding depression for the studied population with scenarios assuming the absence of inbreeding depression.

Materials and methods

Study species and study sites

Pulsatilla alpina (L.) Delarbre (Ranunculaceae) is a perennial, protogynous, andromonoecious hemicryptophyte growing in sub-alpine to alpine habitats in central Europe (Lauber et al., 2018). Several vegetative and/or reproductive shoots emerge from a perennial underground rhizome soon after the snowmelt, from early May to July. Depending on their size and age, individuals produce between zero and ~20 white flowers, each on its own reproductive shoot. Phenotypically male flowers bear only stamens, whereas protogynous hermaphroditic flowers bear stamens and one to a few hundred uni-ovulate pistils. Hermaphrodite and male flowers bear a similar number of stamens (**Chapter I**; Chen and Pannell, 2023). The sex allocation of the species is size-dependent, with larger plants allocating absolutely and proportionally more resources to their female function (**Chapter I**; Chen and Pannell, 2023). Furthermore, small individuals may produce only a single male flower and thus function as pure males in the respective flowering season (**Chapter I**; Chen and Pannell, 2023). Both male and hermaphrodite flowers are predominantly visited by flies, including houseflies and syrphid flies (**Chapter III**; Chen and Pannell, 2022). Ripe fruits (technically achenes) with elongated pappus hair are dispersed by wind in early autumn (Vittoz and Engler, 2007). After fruit dispersal, above-ground vegetative parts senesce, but individuals persist underground for the winter.

We studied how sex allocation affects reproductive success at the flower level during the flowering season of 2022 in a single population of *P. alpina*, located at Solalex in the pre-Alps of Vaud canton, Switzerland (‘Population S1+’; latitude: 46°17′42″N, longitude: 7°09′09″E; elevation: 1758 a.s.l.). The population was located on an open slope of sub-alpine grassland and covering an area with dimensions of about 20 m x 20 m and comprised about 150 mainly small and probably young individuals (following recent establishment after avalanche disturbances and/or herbivory by cattle), each typically producing only a single male or hermaphrodite flower during the season. We set up a 10 m x 15 m temporally fenced plot within the population, enclosing 135 flowering individuals, and removed all the floral buds outside the plot at the very beginning of the flowering season to prevent nearby individuals outside the plot from siring progeny in the plot (thereby improving our ability to assign paternity to phenotyped and mapped individuals).

Flowering phenology

We recorded the flowering state of all the individuals in the population every three or four days throughout the flowering season, from late May to late June 2022, noting the number of flowers, number of stalks, height of the tallest foliar stalk, phenotypic gender, and position of the flowering individuals. Flowers were individually labeled with a paper tag. For each flower and date, we recorded its sexual stage in terms of seven and five categories for hermaphrodite and male flowers, respectively (see Appendix S1 for a detailed description of the categories). We also photographed each flower at the F_2 female stage and the M_1 male stage for hermaphrodite and male flowers, respectively, and later counted the number of stamens on the basis of the photographs (see details below). The flowering date for each flower was calculated as the date of its opening. Around three weeks after the end of the flowering season, all the flowers with developing fruits were enclosed in a paper bag until the end of the growing season (early August), at which point all the seeds were collected.

Manipulation and quantification of floral sex allocation

To amplify variation in male allocation among flowers (for a wide base on which to estimate the shape of the male gain curve), we conducted stamen-removal manipulations throughout the flowering season, as has similarly been done in other studies (e.g., Yund 1998; Johnson and Yund 2009; Aljiboury and Friedman 2022). Specifically, at each time of manipulation, we randomly selected about a quarter of the hermaphrodite flowers in their F_0 stage and removed 100% or 50% of their stamens (treatments SR_{100} or SR_{50}, respectively). Similarly, we removed 50% of the stamens of about a quarter of male flowers in their M_0 stage (treatment SR_{50}). Note that the manipulations were conducted before the anthers dehisced to avoid causing intra-flower selfing. All stamens of SR_{100} flowers were carefully removed by a tweezer, which rendered hermaphrodite flowers functionally female. As a result of our stamen removal treatments, the population comprised a broad mix of morphologically hermaphrodite, male, and female flowers.

We counted the number of pistils and stamens produced in each flower and calculated the functional gender (Lloyd 1980; see below) for each of the flowers in the population. We could easily quantify female allocation for each flower at the end of the season, because all pistils remain on the floral stalk, regardless of whether the associated ovule is fertilized or not. We counted the actual number of stamens for 15 flowers in vivo (N_{actual}) as well as with the aid of photographs (N_{photo}), and we used the regression $N_{actual} = 1.66 \times N_{photo} + 27.2$ ($r^2 = 0.654$) (Chapter I; Chen and Pannell, 2023) to calibrate our stamen counts based on photographs.

We calculated functional gender (femaleness) for each flower following Lloyd (1980), as

$$G_i = \frac{f_i}{f_i + Em_i},$$

where f_i and m_i are the numbers of pistils and stamens, respectively, produced by the ith flower, and $E = \sum f_j / \sum m_j$ is an equivalence factor that accounts for the fact that the total number of genes transmitted through male and female functions must be equal at the population level (with m_j and f_j summed over all flowers in the population).

Estimates of female reproductive success

To estimate the seasonal contribution of each flower to female reproductive success, we collected seeds of all the hermaphrodite flowers that had not been aborted by the end of the flowering season and sorted them into unfertilized, predated, and mature seed categories based on their morphology, following the same method used by Chen and Pannell (2022) in **Chapter III**; note that the sum over all three categories represents a flower's total pistil number. We then calculated components of reproductive success both for our estimated value of inbreeding depression (d) as well as for the assumption that $d = 0$; the comparison of reproductive success estimates under these two scenarios allowed us to infer the extent to which gain curves are sensitive to inbreeding depression. We calculated female reproductive success for our estimate of d as the number of mature outcrossed seeds plus $(1 - d)$ times the number of mature seeds produced by selfing. We calculated female reproductive success for $d = 0$ as the number of mature seeds in which both selfed and outcrossed seeds contributed equivalently to the next generation.

Estimates of male reproductive success

To estimate the seasonal contribution of each individual to male reproductive success, we used variation at ten microsatellite markers to assign paternity to mature seeds after quantifying the female component of fitness. Leaf samples of all flowering individuals were collected in July 2022 at the end of the flowering season and dried in silica gel prior to DNA extraction. Up to ten mature seeds for all seed families were arbitrarily selected for each sampled flower for DNA extraction. To soften the achenes for DNA extraction, we first placed them on wet filter paper in a Petri dish for a week at 4ºC, then removed the embryo. Total DNA was extracted from the leaves and seed samples using the BioSprint 96 DNA Plant Kit (Qiagen, Germany) according to the manufacturer's instructions and eluted in 100 µl of distilled water. Leaf and seed samples were further diluted to 1/100 and 1/20 with distilled water, respectively, to avoid interference with secondary metabolites with potential DNA polymerase activity.

PCR amplification was carried out in a final volume of 10 µl, including 5 µl of 2× Multiplex PCR Master Mix (Qiagen, Germany), 2 µl of diluted DNA, 1 µl of distilled water, and 2 µl of multiplex containing variable primer concentrations (Appendix S2). The ten microsatellite markers, which were selected from 15 candidates developed by ecogenics GmbH (Balgach, Switzerland), were grouped into three multiplexes (Appendix S2). Thermal cycling was performed in a TProfessional Standard Thermocycler (Biometra GmbH, Göttingen, Germany) as follows: 95 °C for 15 min; 36 and 41 cycles for leaf and seed samples, respectively, at a temperature of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 45 s; and a final step at 72 °C for 30 min before cooling down to 4°C. PCR products were analyzed by capillary electrophoresis on an ABI3100 Genetic Analyzer (Applied Biosystems), with an internal size standard GeneScan-350 LIZ. Fragment length analyses and scoring were performed with GeneMapper v 6.0 (Applied Biosystems).

Fathers were assigned to all seeds for which more than five loci were genotyped from among the 135 flowering individuals with Cervus v 3.0.7 assuming a confidence level of 80% and an error rate of 0.018. To further improve paternity assignment, individuals whose flowering timespan did not overlap with the focal hermaphrodite flower were excluded from the list of candidate fathers for each of the seed families genotyped. We calculated male reproductive success for our estimate of d as the siring number of outcrossed seeds plus $(1 - d)$ times the siring number of seeds produced by selfing. We calculated male reproductive success for $d = 0$ as the siring number of seeds in which both selfed and outcrossed seeds contributed equivalently to the next generation. Note that each seed has two copies of genes each derived from one mother and father, thus the total female and male reproductive success of the population should be the same.

Because we genotyped the same number of seeds (ten) across seed families with different numbers of mature seeds, we calculated male reproductive success for each of the two inbreeding scenarios by multiplying the siring seed number of the father with the mature seed number divided by the number of seeds genotyped for each mother. The seasonal male reproductive success was the same at the individual level and the flower level in individuals producing only one flower, whereas it was calculated only at the individual level for individuals producing more than one flower. In addition, pollen dispersal distance was calculated as the distance between the assigned mother and the father for outcrossed seeds. Lastly, the selfing rate, which included autonomous, facilitated, and geitonogamous components of selfing, was calculated for each seed family by dividing the number of selfed seeds by the number of successfully genotyped seeds.

Estimates of inbreeding depression

We estimated inbreeding depression caused by selfing by comparing the inbreeding coefficient for adults in the population (F_{p}) with that of the progeny (F_{o}), following Ritland, 1990. This approach assumes that a reduction of F from the progeny to the parental generations is due to differential mortality of selfed progenies throughout life stages. The parental inbreeding coefficient (F_{p}) and offspring inbreeding coefficient (F_{o}) were estimated from the flowering individuals and genotyped seeds, respectively, genotyped at ten microsatellite markers to estimate the F, using the R package '*hierfstat*' (Goudet, 2005). The population-level selfing rate (s) was estimated by dividing the sum of the inferred number of selfed seeds of each seed family by the total number of mature seeds of the population. Inbreeding depression was then estimated as $d = 1 - [2(1 - s)F_p / s(1 + F_o - 2F_p)]$ (Ritland 1990).

Statistical analysis

We first used the function *Moran.I* in R package *ape* to evaluate the degree of spatial autocorrelation in functional gender across the study site. We used the software SPAGeDi (Hardy and Vekemans, 2002) to assess the spatial genetic structure of the flowering individuals, following the procedure described by Vekemans and Hardy (2004), based on pairwise kinship coefficients between individuals. We conducted Nason's estimator of kinship coefficient ($F_{(r)}$) (Loiselle et al., 1995). The average relationship coefficients of the ten microsatellite markers per distance class were estimated and their significance per class was tested with 1000 permutations. We used *Sp* to evaluate the extent of spatial genetic structure, which is defined as: $S_P = \beta / (1 - F_{(1)})$, where β is the regression slope of $F_{(r)}$ on spatial distance, and $F_{(1)}$ is the mean of $F_{(r)}$ among individuals for the first distance class (Vekemans and Hardy, 2004).

We conducted all the following analysis within the R statistical framework v 4.0.3 (R Core Team, 2021). We used a generalized linear mixed model (*glmer* function in R package *lmer*, Bates et al. 2015) to evaluate the dependency of the intra-floral selfing rate, i.e., the autonomous selfing and facilitated selfing, on five floral traits for individuals producing only one hermaphrodite flower. We set the intra-floral selfing rate as a binomial response variable. We standardized pistil number, stamen number, flowering date, stalk height, and tepal length to a mean of zero and a standard deviation of one and set each of the standardized traits as explanatory variables. We set the identity of each flower as a random variable to account for the fact that the genotyped seeds from the same flower were not independent of each other. We evaluated the fit of the linear model with the R package *DHARMa* (Hartig, 2019).

We used three complementary approaches to study how reproductive success depends on the sex allocation at the flower level and on any possible inbreeding depression. We first analyzed the data in terms of univariate fitness-gain curves, relating prospective reproductive success to sex allocation. Second, we used conventional selection gradient analysis with multivariate regression to evaluate the dependency of reproductive success on both the female and male components of allocation (i.e., as two phenotypic traits), including second-order polynomial and interaction terms (Lande and Arnold, 1983). Lastly, we used nonparametric regression with smoothing functions to depict the fitness landscapes of reproductive success in terms of female and male allocations (Schluter and Nychka, 1994; Morrissey and Sakrejda, 2013).

We used four nonlinear least square models (*nls* function in R *stats*, R Core Team 2021) to evaluate the shape of fitness gain curves of female and male functions at the flower level assuming either $d = 0$ or 0.93. We fitted reproductive success to sex allocation in terms of a power function ($y = ax^b$; Charnov 1979; Aljiboury and Friedman 2022), i.e., the number of pistils and stamens for the female and male functions, respectively, including only the individuals with one flower. The exponent of the power function (b) describes the shape of the curve, with $b < 1$ and $b > 1$ implying a saturating or accelerating dependency of reproductive success on sex allocation, respectively.

We used linear regression models (*lm* function in R *stats*, R Core Team 2021) to evaluate the dependency of female, male, and total reproductive success on both female and male allocation under the two scenarios of inbreeding depression at the flower level in individuals with only one flower. We first standardized female and male reproductive success as the mean for individuals and then fitted the standardized reproductive success as a response variable. We standardized pistil number and stamen number to a mean of zero and a standard deviation of one and set linear, quadratic, and interaction terms of the two traits. This approach allowed us to evaluate linear and non-linear, i.e., quadratic and correlational, selection gradients on female and male allocation (Lande and Arnold, 1983; Matsumura et al., 2012). For all quadratic gradients, we multiplied the regression coefficients by two to obtain the correct estimate of stabilizing or disruptive selection (Stinchcombe et al., 2008).

Lastly, we used generalized additive models (*gam* function in R package *mgcv*, Wood 2003, 2004) to evaluate how fitness landscapes of female, male, and total reproductive success depend on female and male allocation under the two inbreeding depression scenarios. We set female, male, and total reproductive success under the two scenarios as a response variable in six separate models, assuming a Poisson error distribution. We set pistil and stamen numbers as explanatory variables with a smoothing term using thin plate splines. We applied the *gam.gradients* function in the R package *GSG* to extract standardized linear, quadratic, and correlational selection gradients from the fitted models and calculated the standard errors and *P* values on the basis of 1000 bootstraps (Morrissey and Sakrejda, 2013).

Results

Functional gender and morphological space of floral sex allocation

In 2022, the 135 flowering individuals in Population S1+ produced a total of 175 flowers, with 104 individuals producing a single flower and 31 individuals producing more than one flower. The proportion of individuals producing one, two, three, four, and five flowers were 0.77, 0.19, 0.03, 0.01, and 0.01, respectively. Prior to the stamen removal manipulations, 46 and 129 flowers were phenotypically male and hermaphrodite, respectively. Stamen removal manipulations created continuous variation among flowers in functional gender (ranging from zero to one in terms of femaleness) and sex allocation (Figure 1B and C), as intended. Individuals were distributed randomly over space in terms of their functional gender (Moran index = 0.003; Figure 1A, $P = 0.50$); the spatial pattern of sex allocation was thus not considered further.

Estimates of female and male reproductive success

Female reproductive success was estimated by sorting 22,612 achenes from 104 seed families; 19 and 6 seed families from 129 hermaphrodite flowers were aborted or missing, respectively. Each seed family produced on average 90.5 ± 55.4 mature seeds (mean ± SD). DNA extraction was conducted on 1,054 mature seeds, with an average of 10.2 ± 1.3 mature seeds per family. A total of 892 seeds that could be genotyped for at least five loci were used for paternity analysis. We were able to assign paternity for 854 seeds to a single most likely father under a relaxed confidence interval (80%), corresponding to a 96% successful assignment rate. We used the results under the relaxed confidence interval for all the following analyses. In summary, we used paternity analysis to estimate male reproductive success for around 9% of the mature seeds in the population. Note that even under a stricter confidence interval (95%), we were able to assign paternity to 65% of seeds, with downstream results yielding a similar pattern for the variables analyzed (see below). On average, individuals sired 69.6 ± 67.3 mature seeds, estimated on the basis of the adjusted male reproductive success ($d = 0$).

Pollen dispersal distance, spatial genetic structure, selfing rate, and inbreeding depression

Pollen dispersal distances for outcrossing were generally short, with an average of 3.16 m separating sire from the dam and 25%, 50%, and 75% of seeds sired by males <1.0 m, < 2.15 m, and < 4.13 m away from the corresponding dam (based on $N = 513$ matings; Appendix S3A). Spatial genetic structure analysis among the 135 flowering individuals showed a significantly negative β value ($b_F = -0.002$, $p < .001$; Appendix S3B). The *Sp* statistic value of the flowering individuals was 0.0022.

A proportion of 0.4 (341 of 854) of genotyped seeds was estimated as having been self-fertilized. The intra-floral selfing rate, calculated for individuals with only one flower, decreased with pistil number ($P < 0.05$) and flowering date ($P < 0.05$) and increased with floral stamen number ($P < 0.001$; $N = 53$; Figure 2). The inbreeding coefficient of the parents (F_p) and offspring (F_o) were 0.028 and 0.221, respectively. This implies a level of inbreeding depression of 0.93, as determined by the two-generation equilibrium estimator (Ritland, 1990).

One-dimensional female and male flora fitness gain curves

We estimated the shape of the male and female gain curves on the basis of reproductive success estimates for 88 parents that produced only one non-aborted flower. Means (± SD) of female, male, and total reproductive success were inferred to be 65 ± 58.4, 57.8 ± 51.4, and 122.9 ± 88.2, respectively under the assumption of $d = 0$, and 41.4 ± 48.5, 34.2 ± 41.2, and 75.6 ±60.9, respectively, under $d = 0.93$ (Figure 3). The exponents *b* (± SE) for the female fitness gain curves were 0.85 ± 0.16 (non-significantly different from 1.0, Figure 4A) and 1.9 ± 0.29 (significantly > one, Figure 4C) under the assumption of $d = 0$ and $d = 1$, respectively, pointing to linear and accelerating gain curves. The corresponding exponents *b* for the male fitness gain curves were 0.52 ± 0.27 (Figure 4B) and 0.47 ± 0.37 (Figure 4D), respectively. Although neither of these two estimates was significantly different from one, they trend toward a saturating gain curve for male function.

Linear, quadratic, and correlational selection gradients on female and male allocation

Female reproductive success was an accelerating function of allocation for *d* = 0.93 but would have been linear for *d* = 0. Male reproductive success was a largely saturating function of allocation for both *d* = 0.93 and *d* = 0 (Figure 5A, E, G, K, and Appendix S4). These patterns conform to the results based on univariate regression with a power function. Nonetheless, our results point towards disruptive selection on male allocation in terms of female reproductive success for *d* = 0.93 (Figure 5J and Appendix S4) and to stabilizing selection on female allocation in terms of male reproductive success for *d* = 0 (Figure 5B and Appendix S4). Total reproductive success depended only on female allocation when *d* = 0.93 and would have depended on both female and male allocation for *d* = 0 (Figure 5C, F, I, and L and Appendix S4). Lastly, we were not able to detect any correlational selection on female and male allocation, i.e., all correlational selection gradients were not significantly different from zero (Appendix S4).

Fitness landscapes for female, male, and total reproductive success as a function of sex allocation

Visualization of predicted fitness landscapes of female, male, and total reproductive success on female and male allocation for *d* = 0.93 and *d* = 0 are presented in Figure 6. Deviance explained by fitted models for female, male, and total reproductive success was 80.2%, 58.3%, and 53.7%, respectively, for *d* = 0.93, and 85.7%, 63.3%, and 67.5%, respectively, for *d* = 0. Standardized linear, quadratic, and correlational selection gradients estimated using the *GSG* package are presented in Appendix S5. Note that we detected evidence for correlational selection gradients for female, male, and total reproductive success when *d* = 0.93 (Appendix S5).

Discussion

In the present study, we measured female, male, and total reproductive success of phenotypes with different sex allocation, taking into account the effects of the mating system and inbreeding depression. Our analysis revealed evidence of dependency of reproductive success on the sex allocation to the opposite sex and interactions between the two sexes. This important insight derives from the novel conception of a fitness landscape in two dimensions rather than in terms of the simpler one-dimensional sex-allocation gain curves that have usually been invoked to understand floral strategies. Our analysis also shows that the mating system and inbreeding depression greatly alter the mapping of reproductive success on sex allocation. Below, we first discuss potential mechanisms for the observed patterns, before considering their implications for an understanding of the gender diphasy and andromonoecy in *P. alpina* and of sex allocation strategies in plants in general.

Mating system, pollen dispersal distances, spatial genetic structure, and inbreeding depression

We found that *P. alpina* possesses a mixed mating system with a short pollen dispersal distance. First, it is not surprising that the actinomorphic, fly-pollinated, and self-compatible flowers of *P. alpina* with several whorls of pistils and stamens are prone to self-fertilization as a result of overlapping between the two sexual stages, lack of spatial separation between pistils and stamens, and pollinator foraging for pollen reward within a flower (Devaux et al., 2014; Inouye et al., 2015). Our estimate of the selfing rate for *P. alpina* is comparable to that for other insect-pollinated perennial herbs (references in Whitehead et al., 2018), although our value is likely a lower bound for the species more generally because most individuals in the study population produced only a few flowers, whereas *P. alpina* plants often produce many flowers simultaneously, so that geitonogamous selfing is likely frequent, too. Furthermore, the mean and 50 percent quantile of pollen dispersal distance were found to be three and two meters, respectively. Although it has been suggested that pollen dispersal distance in herbaceous species is in general short (references in Tomaszewski et al., 2018), the considerably short pollen dispersal distance in *P. alpina* is also likely a consequence of fly pollination and high population density (Levin and Kerster, 1969; Rader et al., 2011; van Rossum et al., 2011). Certainly, the considerable rates of self-pollen transfer and short distances over which outcross pollen is dispersed points to as likely strongly flattening male fitness gain curve for *P. alpina*. In contrast, the fact that we found very weak spatial genetic structure ($Sp = 0.002$) among adult individuals, an unusual finding for herbaceous, animal-pollinated species with mixed mating (Vekemans and Hardy, 2004), suggests that seed dispersal is very effective and that the female fitness gain curve is much less saturating than the male one (but the discussion on fitness gain curves at the flower level, below).

We found a strong inbreeding depression close to one ($d = 0.93$) estimated by the inbreeding coefficients of two generations (Ritland, 1990). The estimated inbreeding coefficient of the flowering parents was close to zero whereas that of the progenies was 0.2, indicating a strong fitness disadvantage to selfed progeny, as expected and commonly observed for perennial plants (Morgan, 2001; Goodwillie et al., 2005; Scofield and Schultz, 2005; Angeloni et al., 2011). The inbreeding depression in *P. alpina* likely manifests at the early seedling and non-flowering stages, as the mortality rate was the highest in those stages and became independent of age and size upon maturation in a congenic species (Edelfeldt et al., 2019). We may presume that the high level of inbreeding depression combined with substantial selfing in *P. alpina* means that there is strong ongoing selection for improved outcrossing mechanisms.

Implications of the mating system and inbreeding depression on fitness gain curves

We found that the presence of a strong inbreeding depression (i.e., when $d = 0.93$) not only largely reduced the means and variances of absolute female, male, and total reproductive success, but also altered the shape of the distribution to be more ridged, especially for total reproductive success. The results imply that the selfing rate is likely not uniform across phenotypes with different suites of sex allocation. Indeed, we found that the intra-flora selfing rate depends negatively and positively on floral sex allocation to female and male functions, respectively, pointing to a seed discounting in female reproductive success. Producing more pistils and fewer stamens within a flower likely reduces the proportion of pistils close to stamens and subject to selfing as a consequence of increased herkogamy found in other species with a fixed number of sexual organs (e.g., Brunet and Eckert 1998; Takebayashi et al. 2005). The present study demonstrates the first evidence of how the mating system depends on the sex allocation of the flowers, and the pattern found in *P. alpina* may be general in other species producing flowers with multiple whorls of sexual organs, e.g., in most of the basal angiosperms and basal eudicots (Ronse De Craene et al., 2003; Kim et al., 2005).

Our results demonstrate that the dependency of the selfing rate on floral sex allocation and the consequence of seed discounting further led to an accelerating female gain curve, a mostly negative dependency of female reproductive success on male sex allocation, and a fitness valley of intermediate female sex allocation and high male sex allocation in the landscape of female reproductive success. When $d = 0$, female reproductive success depended on only the female function linearly but not on the male function because both outcrossed and selfed seed contributed to the female reproductive success, i.e., no seed discounting caused by increasing male sex allocation. However, when $d = 0.93$, flowers with low to intermediate female sex allocation suffered the most from seed discounting and thus the female gain curve became accelerating and the female reproductive success was mostly negatively dependent on the male sex allocation, which points to potential sexual interference between the two sex functions within a flower (Barrett, 2002). Our results stress that considering the effects of the mating system is critical and necessary when measuring the dependency of female reproductive success on sex allocation.

Conversely, we found that the degree of inbreeding depression likely did not substantially affect the dependency of male reproductive success on male sex allocation whereas it altered the dependency on female sex allocation. Firstly, the dependency of the male reproductive success on male sex allocation was mostly saturating independent of the degree of inbreeding depression, which provides novel evidence to the general expectation of insect-pollinated species (Charnov, 1982; Campbell, 2000). Nonetheless, our multivariate analysis showed a significantly quadratic and a trend of negative dependency of the male reproductive success on the female sex allocation when $d = 0$ and 0.93, respectively. When $d = 0$, the male reproductive success firstly increased with the female sex allocation likely as a result of an increased number of seeds sired by intra-floral selfing. Whereas, the male reproductive success started to decrease after a certain threshold of female sex allocation likely as a result of increased sexual interference due to delayed onset of the male function (Chapter I; Chen and Pannell, 2023), which may also explain the negative trending when $d = 0.93$. Interestingly, we found a much more pronounced negative dependency of male reproductive success on the female sex allocation in a supplementary analysis using only H flowers (Appendix S6 and S7), which conforms to our expectation of sexual interference by female sex allocation on male reproductive success.

Most of the sex allocation models assumed a fixed selfing rate for the population concerned and thus how the dependency of the mating system on sex allocation affects sex allocation strategies and the evolution of sexual systems has been largely overlooked. For instance, it is suggested by Lloyd 1987 and Brunet 1992 that only the autonomous selfing rate but not the facilitated and geitonogamous selfing rate determines the sex allocation in hermaphroditic plants. On the contrary, the model of De Jong et al. 1999 showed that when the selfing rate depends positively on male sex allocation and the inbreeding depression is high, a dioecious sexual system or size-dependent sex allocation strategy may evolve given that the male gain curve is linear or saturating, respectively. Our results found in *P. alpina* are mostly in accordance with the model as the species possesses a size-dependent sex allocation strategy in which sex allocation to the female function increases with size (Chapter I; Chen and Pannell, 2023). Nonetheless, whether the dependency found in one-flowered individuals holds in individuals with multiple flowers deserves further investigation.

From fitness gain curves to fitness landscapes: adaptive implications on gender-diphasy and andromonoecy

We found that the dependency of reproductive success on sex allocation to the opposite sex function as a result of sexual interference and inbreeding depression led to a sophisticated fitness landscape of total reproductive success with multiple peaks and valleys. A pattern could not be depicted and predicted by conventional fitness gain curves using univariate functions, providing us with new insights into the evolution of sex allocation strategies and sexual systems. Below, we discuss the implications of our results in *P. alpina* on gender-diphasy and andromonoecy from a conceptual point of view.

The resource status a plant possesses determines the absolute sex allocation to be allocated to the two sex functions, thus a small plant may not be able to produce the same amount of stamens and/or pistils as a large plant does. Assuming there is a strictly linear trade-off between the two sex functions under a certain amount of resource (R) for reproduction (lines a, b, and c for R_a, R_b, and R_c representing small, medium, and large plants, respectively, in Figure 7A), the ratio of the cost of one unit of the female to male function determines the slope of the trade-off line which should be a constant in a species and be smaller than -1 because a female unit is usually costlier than a male unit. An individual can produce different numbers of female and male units along the trade-off line if all the resource is used. Alternatively, it can produce any less number of units below the trade-off line in which there is resource unused. As a result, such a trade-off line dictates the boundary on a fitness landscape that an individual can explore given the amount of resource, and thus an individual can explore a wider landscape with a greater amount of resource. Within such a boundary, an individual is expected to produce female and male units that maximize the total reproductive success, i.e., the fitness peaks in the landscape.

The topography of the fitness landscape revealed by our results immediately provides a compelling explanation for both gender diphasy in *P. alpina* and for its andromonoecious sexual system. First, individuals are likely to adopt different sex allocation decisions according to their resource status during their life span, leading to a so-called size-dependent sex allocation strategy or shifts between two gender phases. Given a fitness landscape of total reproductive success under a strong inbreeding depression as the case in *P. alpina*, individuals with a limited amount of resource, e.g., R_a, should allocate most of the resource to the male function and function as a male. Moreover, individuals with a resource equal to or less than R_b is favored to produce a highly male-biased flower or even a male-only flower instead of using all the resource. On the other hand, individuals with a substantial amount of resource, e.g., R_c, should produce a high number of both female and male units, i.e., a hermaphroditic flower. As an individual grows larger, it is likely to acquire more resource for reproduction. Thus an individual may shift its gender from male-phase to hermaphrodite-phase according to its resource status, a phenomenon so-called 'gender-diphasy' that has been found in *P. alpina* (Chapter I; Chen and Pannell, 2023) and also other perennial herbs (Freeman et al., 1980; Schlessman, 1988).

The landscape topography revealed by our results also points to an explanation for andromonoecy in *P. alpina*. As plants are highly modular, the same mechanism discussed above may play a similar role in the sex allocation decision for each floral within a hermaphroditic individual to maximize the output of reproductive success assuming the total flower number does not alter the landscape. For instance, with a given amount of resource pool R_T that ought to be shared by several flowers, producing some male flowers using the resource left from producing hermaphroditic flowers will likely enable an individual to flexibly adjust its sex allocation based on the resource pool and yield the highest reproductive success (Spalik, 1991; de Jong et al., 2008). As a consequence, the individual may produce a mixture of male and hermaphroditic flowers, a sex allocation strategy characterized as 'andromonoecy'. Again, such a sex allocation strategy has been found in not only *P. alpina* (Chapter I; Chen and Pannell, 2023) but also in many other perennial herbs (Pellmyr, 1987; Schlessman, 2010; Peruzzi, 2012). Lastly, given that sexual interference between the two sex functions, a strong inbreeding depression, and limited resource for reproduction with a higher cost of the female function may be common in other hermaphroditic species as well, the present study thus presents a plausible and general explanation for gender-diphasy and andromonoecy.

Implications on the general advancing of sex allocation studies

We found a significant interaction between the two sex functions in determining the fitness landscape of total reproductive success when $d = 0.93$, indicating that fitness gain curves are not a fixed function independent of absolute sex allocation. Figure 7B depicting a graphical perspective on these effects shows that the relationship between total reproductive success and relative male sex allocation along the hypothetical trade-off lines of different resource statuses is highly variable, which indicates that the dependency of both female and male reproductive success on the sex allocation can vary considerably with the different absolute amount of resource. In another word, the shapes of the fitness gain curves are not independent of the absolute amount of resource, which is likely a consequence of complex sexual interference and not a direct effect of size or resource (Klinkhamer et al., 1997). This has a great implication because, to our knowledge, most of the models studying sex allocation in hermaphroditic plants assumed a fixed exponent of the gain curves regardless of the absolute amount of resource (Charnov, 1979; Charlesworth and Morgan, 1991; Klinkhamer et al., 1997; Sakai, 2000; Zhang and Jiang, 2002), which is likely not the case in *P. alpina* and other species as well. Thus, our results point to a gap that deserves further investigation both theoretically and empirically.

Sex allocation theory has provided us with a powerful heuristic for understanding sexual-system evolution, but it has been difficult to apply to hermaphroditic populations because of the need to measure sex allocation in each of the two sexual functions in the same currency. Indeed, in the first theoretical application of sex-allocation theory to explain the maintenance of combined versus separate sexes, Charnov et al., 1976 suggested that selection might be particularly favorable to hermaphroditism when resources invested into the two sexual functions do not overlap, e.g., if they draw on different currencies, or occur at different times. Yet, almost ironically, this explanation violates the basic tenet of sex-allocation theory, which requires the formulation of male and female functions in the same currency as a single point along a one-dimensional continuum. In depicting the dependency of reproductive success on sex allocation in terms of a simple power function, often regardless of the potential absolute resource status of the individuals concerned, and in ignoring the dependency of reproductive success through one sex on the sex allocation of the opposite sex, sex allocation theory has been difficult to apply to hermaphrodite populations. In contrast, our approach somewhat bypasses the need to seek a common currency between the sexes – because a two-dimensional landscape perspective allows allocation to each sexual function to be cast independently of one another.

Finally, we wonder whether the application of classical sex allocation theory might have been overlooking dimensions of the complexity of hermaphroditic strategies that underlie important aspects of sexual-system evolution. By considering the fitness effects of male and female allocation beyond their tradeoffs with one another, we have been able to decompose the fitness consequences of sex allocation more finely, allowing us to formulate explanations for variation within and among individuals in sex allocation that would have been difficult to do on the basis of a one-dimensional perspective. We anticipate that this perspective may contribute more generally toward understanding the complexity of hermaphroditic sex allocation. In an important sense, it might offer a rescue line to a body of powerful theory that has, however, been criticized for being too difficult to apply to the messy world of the reproduction of hermaphrodites (Emms, 1993; Campbell, 2000; Thomson, 2006; Schärer, 2009).

Acknowledgments

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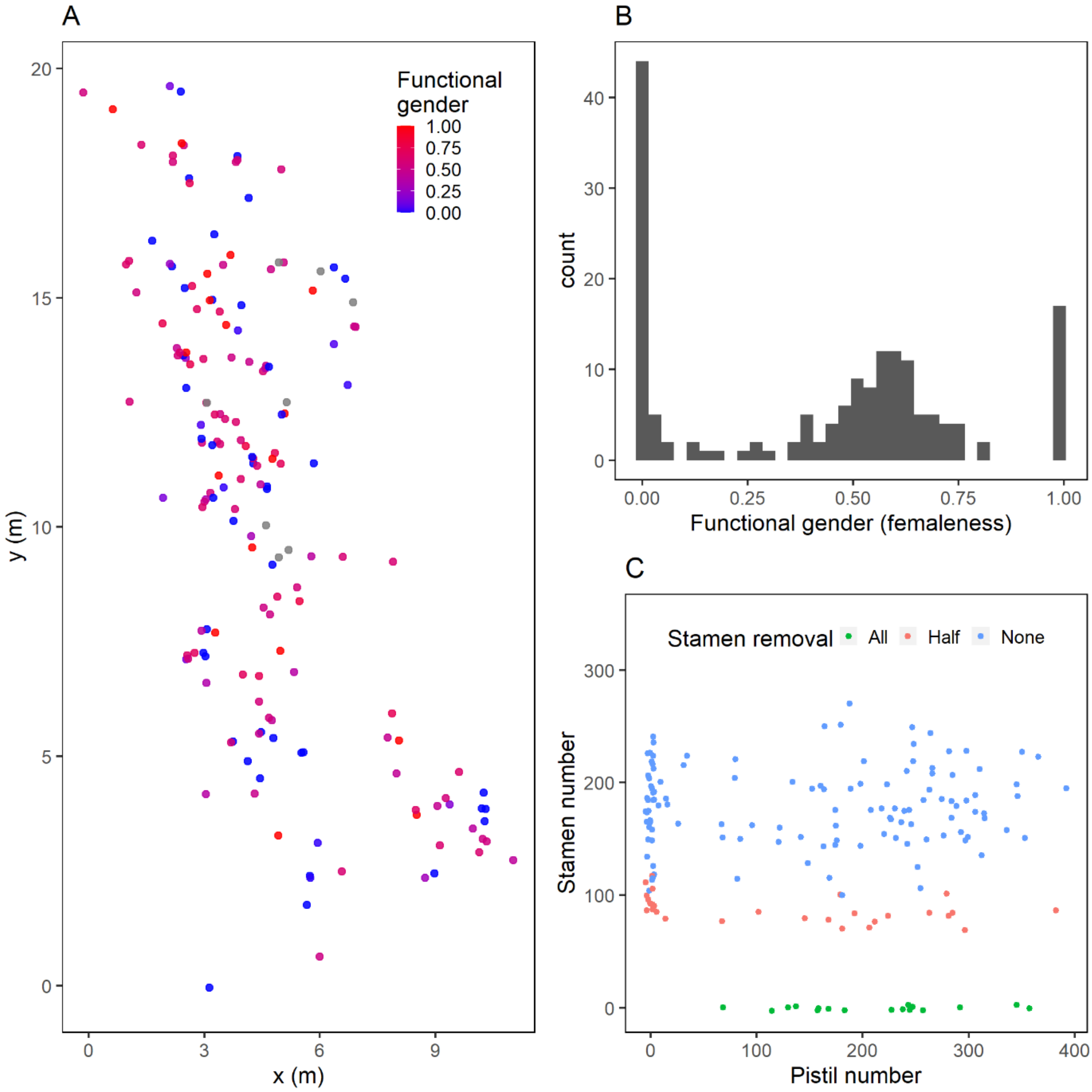


Figure 1. (**A**) Position of the flowers and their functional gender calculated in femaleness within the plot in Population S1+ (*N* = 175). Each point represents one flower. Points of the flowers from multiple-flowered individuals are jittered to avoid overlapping. The grey points represent missing data (*N* = 8). (**B**) Histogram of the functional gender of the flowers. A functional gender of zero refers to a phenotypically male flower, whereas that of one refers to a phenotypic female flower (created by stamen removal treatments). (**C**) Morphological space of stamen and pistil number of the flowers after stamen removal treatments. Green, orange, and blue points represent all-stamen-removed, half-stamen-removed, and intact flowers, respectively (*N* = 17, 28, and 130 respectively).

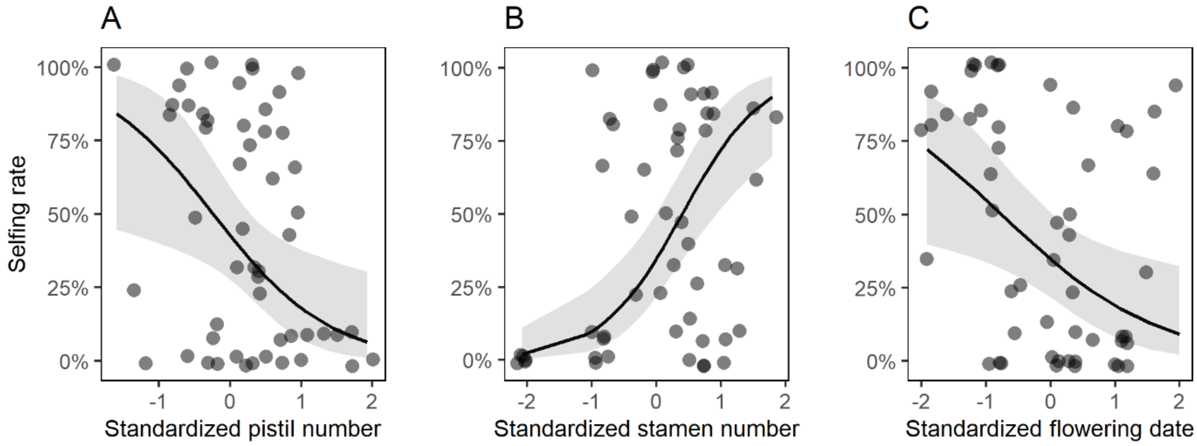


Figure 2. Plots showing the effect of pistil number (**A**), stamen number (**B**), and flowering date (**C**) on the intra-floral selfing rate estimated by a multivariate *glmer* model. Each point represents the selfing rate of one flower (N = 53 H flowers). The shaded ribbon indicates the 95% confidence interval of the regression lines.

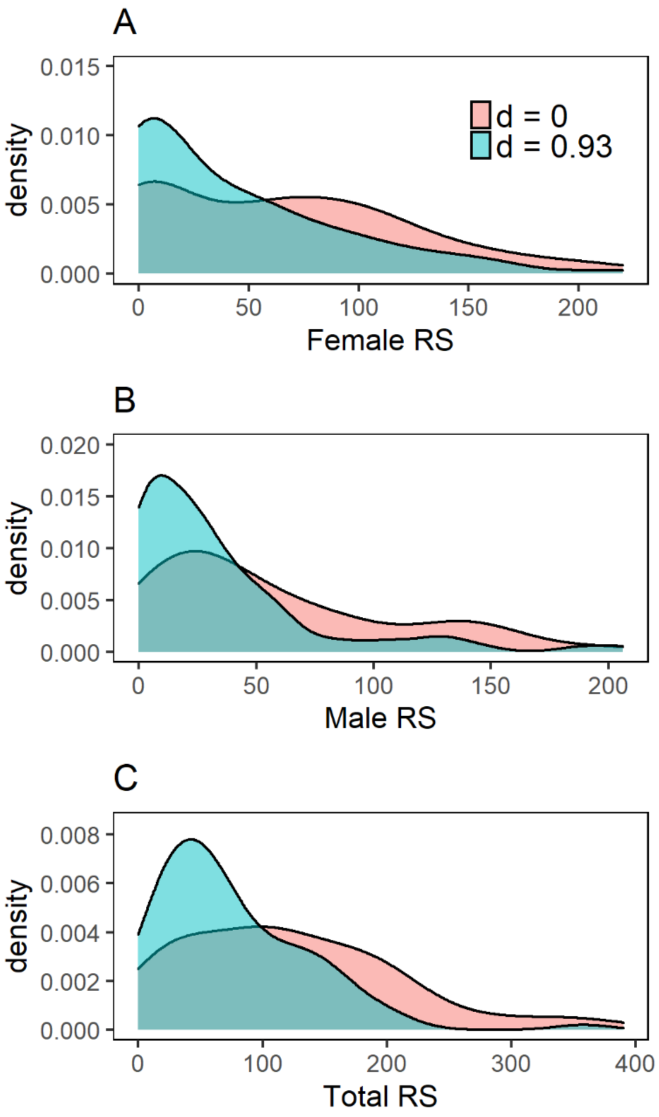


Figure 3. Density plots of female (**A**), male (**B**), and total (**C**) reproductive success of one-flowered individuals under two scenarios of inbreeding depression (*N* = 88). reproductive success represents the actual number of seeds sired and/or produced. Scenarios of an inbreeding depression of zero and 0.93 were shown in red and blue, respectively.

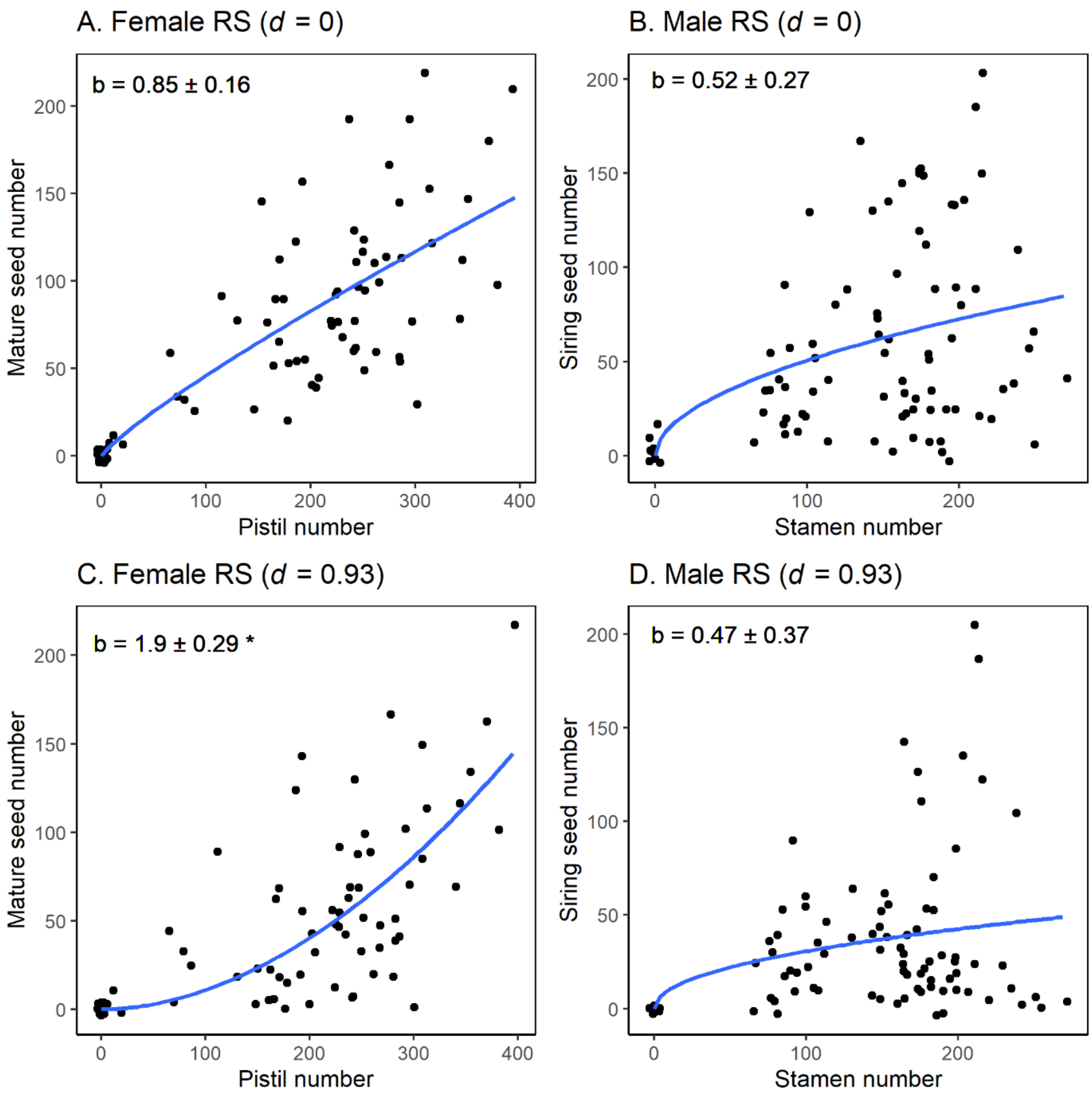


Figure 4. Fitness gain curves of female (left-hand panels) and male functions (right-hand panels) at the floral level under the condition of *d* = 0 (upper panels) and *d* = 0.93 (bottom panels). Each point represents one individual with one flower (*N* = 88). The points were jittered to avoid overlapping. The shape of the gain curves (blue lines) was estimated by fitting exponential curves (see materials and methods for details) and the exponent *b* is shown in the figure with the standard error. An asterisk denotes that the curve was significantly non-linear.

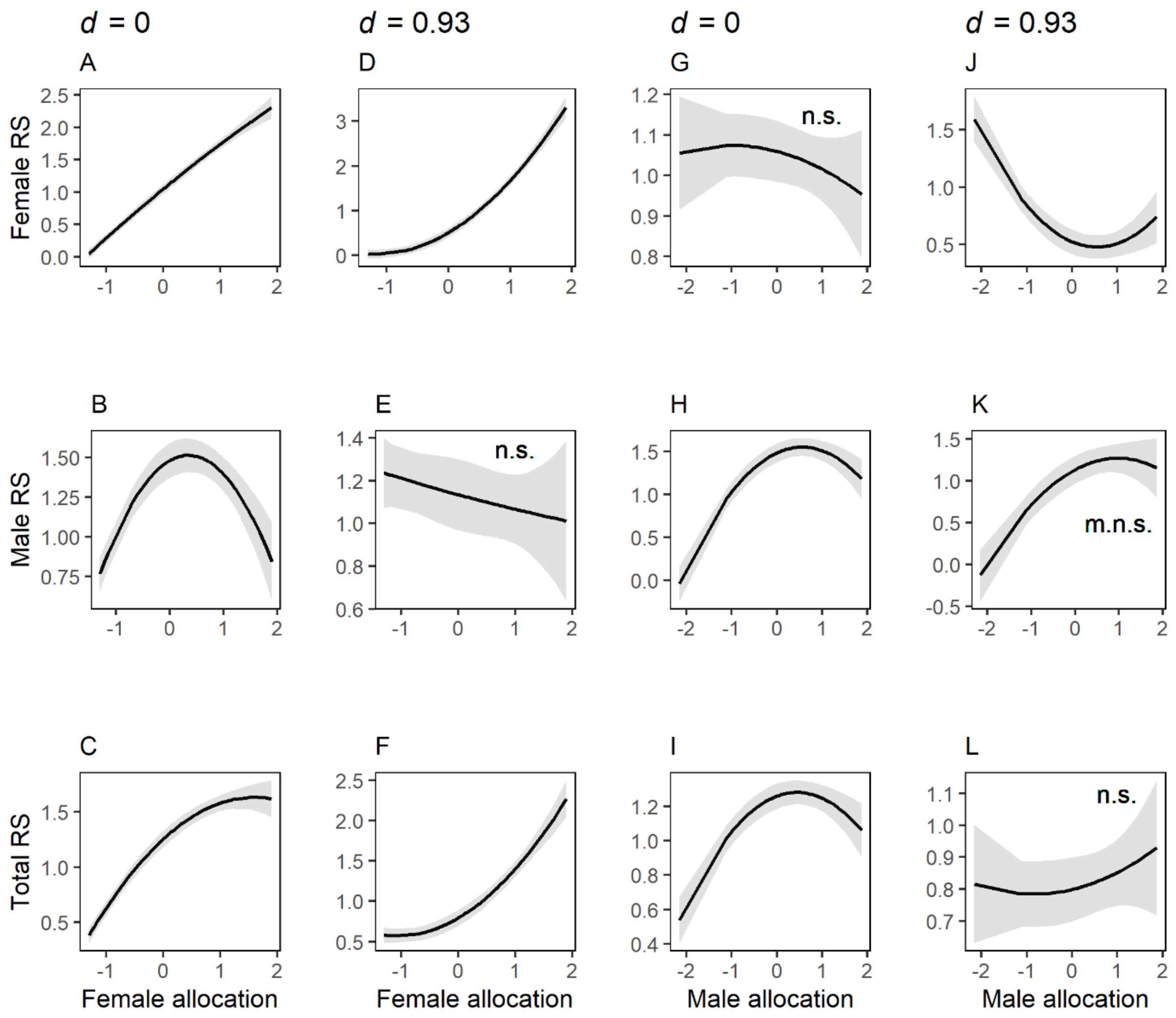


Figure 5. The dependency of female (upper panels), male (middle panels), and total (bottom panels) reproductive success on female and male allocation under the condition of *d* = 0 (Panels **A – C** and **G - I**) and *d* = 0.93 (Panels **D – F** and **J – L**), estimated by selection gradient analyses (*N* = 88, see materials and methods for details). Female and male allocation refers to standardized pistil and stamen number of each flower, respectively. The shaded ribbon indicates the standard error of the regression curves. Panels showing non-significant and marginally non-significant dependency of reproductive success on the sex function are labeled with ‘n.s.’ and ‘m.n.s.’, respectively.

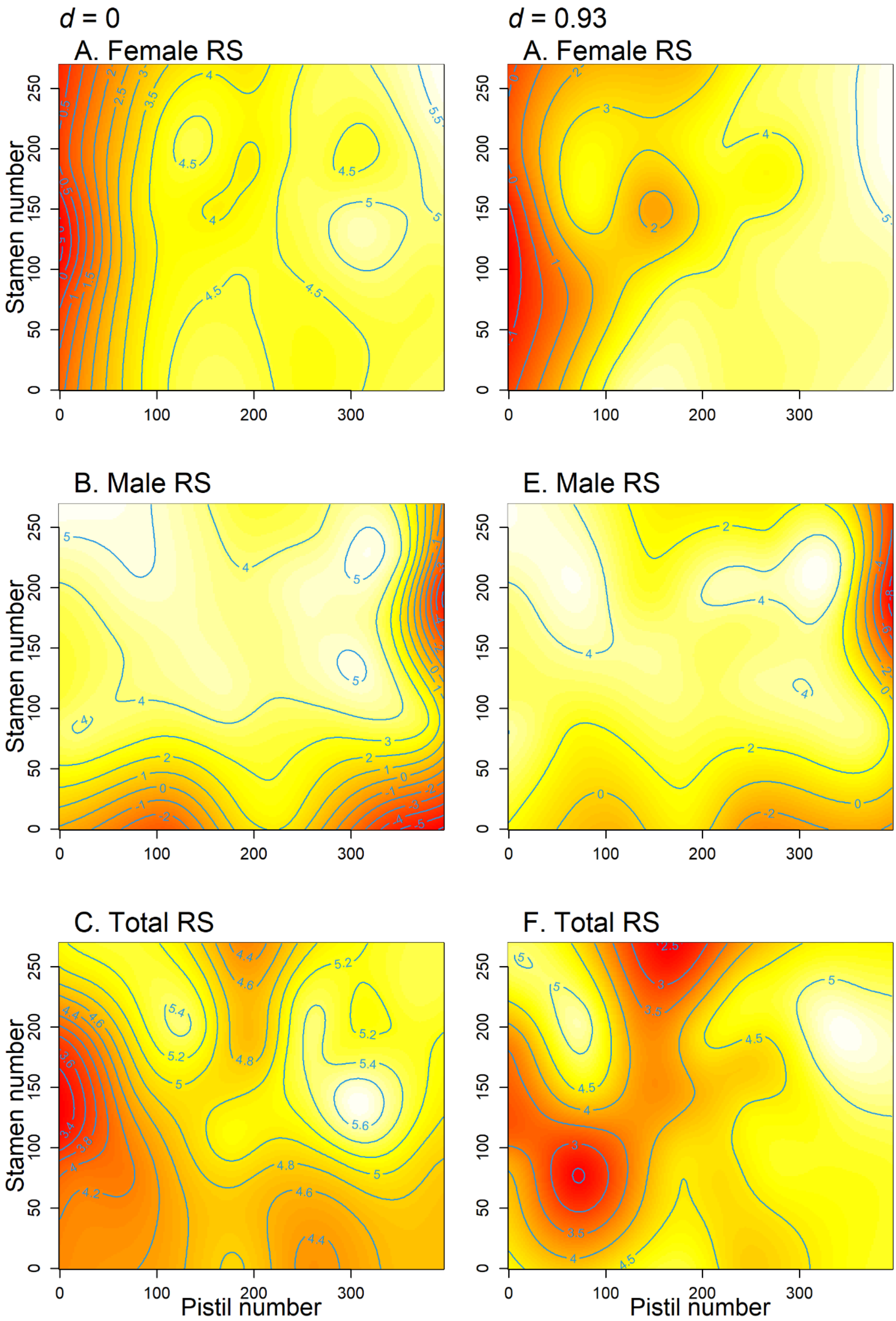

d = 0
d = 0.93
A. Female RS
A. Female RS
B. Male RS
E. Male RS
C. Total RS
F. Total RS
Stamen number
Stamen number
Stamen number
Pistil number
Pistil number
0
50
100
150
200
250
0
100
200
300

Figure 6. Representations of the fitness landscape for female, male, total reproductive success as a function of pistil and stamen number in a flower under the scenario of $d = 0$ (Panels **A - C**) and $d = 0.93$ (Panels **D - F**), predicted by generalized additive models (*gam*) using 88 individuals with a single flower. The color gradient from red to white represents low to high predicted reproductive success.

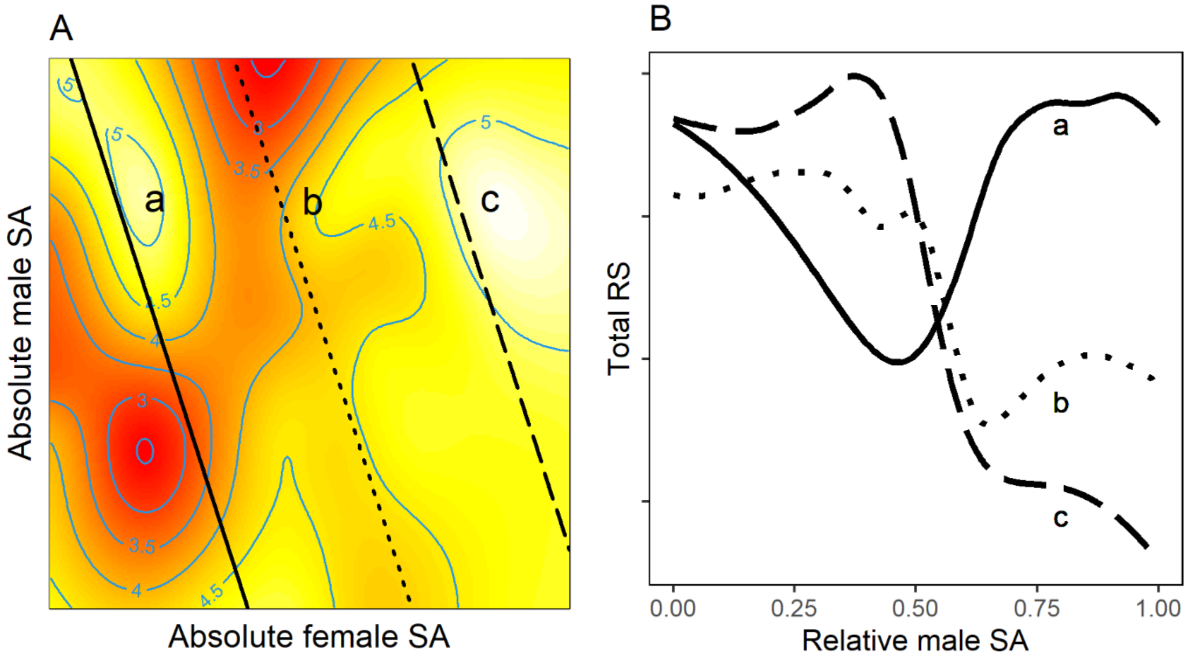


Figure 7. Conceptual figures demonstrating how the resource status of an individual affects the exploration of the fitness landscape of sex allocation based on the study of *P. alpina*. (**A**) Fitness landscape of total reproductive success on absolute sex allocation to female and male functions when $d = 0.93$ (adapted from **Figure 6F**). Line a, b, and c depict the conceptual linear trade-off between the two sex functions on a fixed amount of resource for reproduction (R_a, R_b, and R_c, respectively; $R_a < R_b < R_c$). Note that the slope of the trade-off lines is conceptual because we do not know the actual trade-off ratio of one female and male unit. Individuals with a given amount of resource are only able to explore the left and bottom part of the trade-off line on the fitness landscape. (**B**) Relationship of the total reproductive success and relative sex allocation to the male function along the trade-off lines a, b, and c extracted from (**A**). Relative male sex allocation was calculated by dividing the absolute male sex allocation by the sum of absolute female and male sex allocation.

Supplementary materials

Appendix S1. Categories of sexual stages for hermaphroditic and male flowers.

Sexual stages	Definition
B	Bud stage. From the emergence of the bud before its opening.
F_0	Early female stage. Only applicable to hermaphroditic flowers. From the opening of the tepals till the tepals are fully open.
F_1	Female stage one. Only applicable to hermaphroditic flowers. Tepals are fully open and elongated. Filaments are short and anthers are still fully enclosed.
F_2	Late female stage. Only applicable to hermaphroditic flowers. Tepals are fully open and elongated. Filaments start to elongate and some of the stamens (<5%) may have dehisced. This is the stage at which intra-floral selfing is most likely to happen.
M_0	Early male stage. Only applicable to male flowers. From the opening of the tepals till the tepals are fully open.
M_1	Male stage one. Tepals are fully open and elongated. Filaments are elongated and the anthers have dehisced.
M_2	Late male stage. Tepals are fully open and start to senesce. Filaments are elongated and more than half of the anthers have already dehisced.
E	End of the flowering. Tepals senesced and dropped.

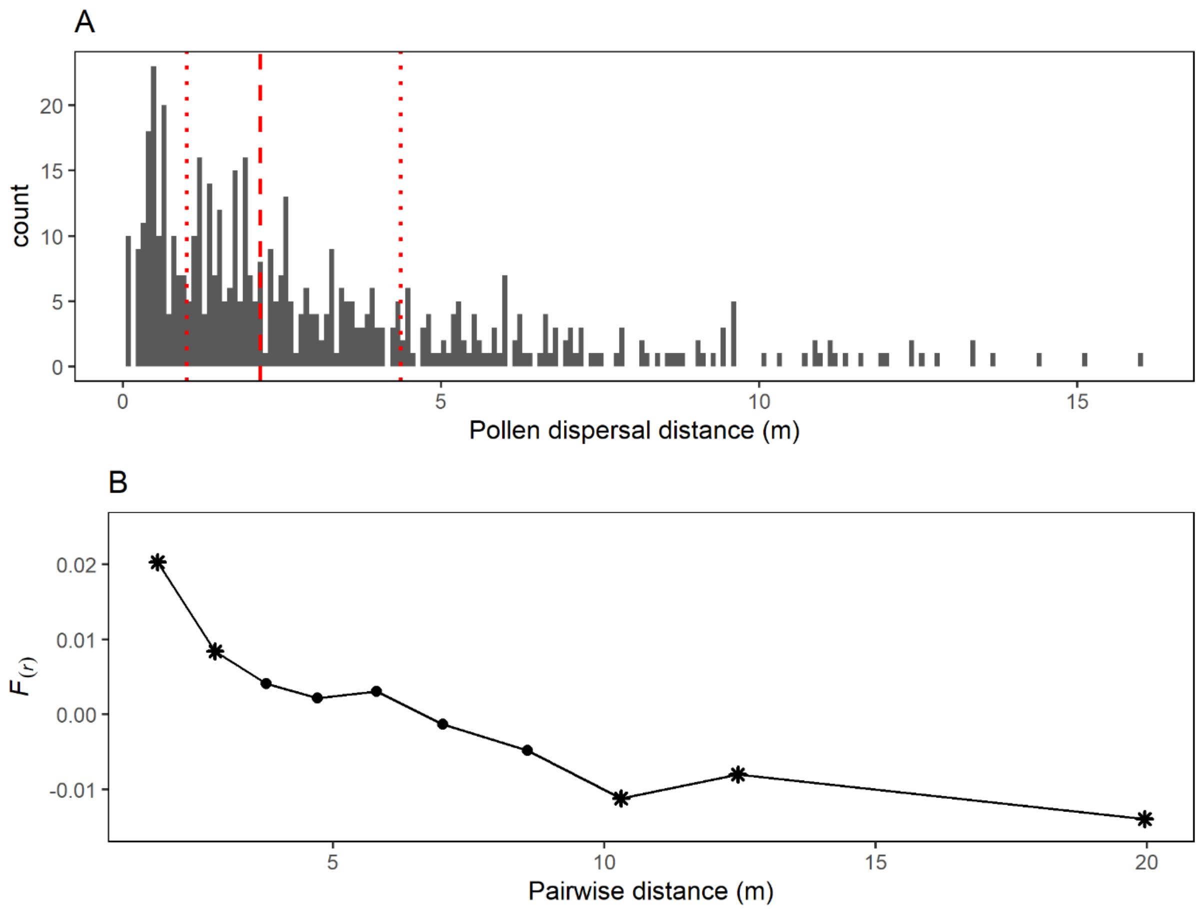
Seven (B, F_0, F_1, F_2, M_1, M_2, E) and five (B, M_0, M_1, M_2, E) categories are applicable to hermaphroditic and male flowers, respectively.

Appendix S2. Characteristics of three primer multiplexes used in the paternity analysis in *P. alpina* based on the parental individuals (*N*=136).

Multiplex	**Locus**	**Primer sequences (5'-3')**	**Repeat motif**	**Size range (bp)**	**T_A (ºC)**	**FC (µM)**	**N_A**	**H_O**	**H_E**	**F_{Null}**
A1	P14	*HEX*-ATCTGACAATCCACCCGTGC ACTCCCACAAGCTTTGTATTTCC	(TC)12(TA)27	179-202	60	0.2	11	0.65	0.8	0.1
	P6	*FAM*-ACACCACTGAGTCTGGCATC CTGGCATGACCGTATGGAGG	(AG)13	212-257	60	0.2	13	0.87	0.81	-0.04
	P7	*HEX*-GCGGAGTTATTGAGGAGAACTG ATGGTGTACCGTTTGAGCTG	(GT)15	123-145	60	0.2	7	0.71	0.73	0.01
	P8	*FAM*-TATCTTGTAACACCCCTCGC ATGTGGCCTCTTATTCAGGC	(ATAC)8	87-108	60	0.2	5	0.72	0.69	-0.02
A2	P1	*ATTO565*-CCAGGGCGGATTTAACAGTC AGGTATCCACTGAATTTTTGTTTTTG	(TA)15	98-123	60	0.4	14	0.75	0.79	0.03
	P13	*ATT0550*-TGAAACTAGGGTCTACCCCG CCCATATTTGGCCGTCAACC	(AT)12	162-176	60	0.1	5	0.51	0.5	-0.01
	P2	*ATTO565*-ATCCTGTTTTAGGCGCTGAC TGTCAAACTTCGCACGGATG	(CT)12	220-246	60	0.1	10	0.77	0.78	0
	P3	*FAM*-AGATAGTGGTGATGGTGGCG CAGTACTCCTGGTGCTAGGC	(TGTT)7	184-201	60	0.2	5	0.43	0.41	-0.03
A3	P5	*HEX*-CGGAGTGCTTGGGATCAAAC TCAAACCTGCCAGAATACTTCC	(CA)13	238-275	60	0.6	10	0.59	0.79	0.13
	P9	*ATTO550*-GTTCCAAAGTACACAATGAAGGC TCCCAGATCTTGTAGACGGTG	(GA)14	212-231	60	0.1	7	0.56	0.58	0.01

Note: Fluorescent tags attached to the 5’ end of the forward primers are indicated in italic.

T_A, annealing temperature; FC, final concentration of each primer in the PCR reaction; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{Null}, estimated frequency of null allele



Appendix S3. (A) Pollen dispersal distance estimated for outcrossed seeds (*N* = 513). The red dashed line represents the median distance whereas the two red dotted lines represent the 25% and 75% quantiles. The mean pollen dispersal distance was 3.16 m. (B) Results of a spatial autocorrelation analysis based on the flowering individuals (*N* = 138), and on the use of Nason's kinship coefficients ($F_{(r)}$). The average kinship coefficients (marked as dots) are positioned along the X-axis at the mean pairwise distance within each of the ten distance classes. The average $F_{(r)}$ values deviating significantly from expected for a random distribution of genotypes are marked in an asterisk.

Appendix S4. Linear (β_i), quadratic (γ_{ii}), and correlational (γ_{ij}) selection gradients on female and male allocation via female, male, and total reproductive success (RS) under the scenario of an inbreeding depression of zero and one. Female and male allocation refers to standardized pistil and stamen number of each flower, respectively.

Notes: n.s. $P > 0.1$, **.** $P < 0.1$, ∗ $P < 0.05$, ∗∗ $P < 0.01$, ∗∗∗ $P < 0.001$

		Female RS		**Male RS**		**Total RS**	
Inbreeding depression scenario	**Selection gradient**	**Estimate (SE)**	***P* value**	**Estimate (SE)**	***P* value**	**Estimate (SE)**	***P* value**
ID = 0	β female allocation	0.72 (0.06)	***	0.19 (0.09)	*	0.47 (0.06)	***
	β male allocation	-0.031 (0.07)	n.s.	0.24 (0.10)	*	0.097 (0.07)	n.s.
	γ female allocation	-0.066 (0.07)	n.s.	-0.55 (0.10)	**	-0.30 (0.07)	*
	γ male allocation	-0.029 (0.05)	n.s.	-0.43 (0.08)	**	-0.22 (0.05)	*
	γ female allocation, male allocation	0.19 (0.07)	n.s.	-0.084 (0.11)	n.s.	0.059 (0.07)	n.s.
ID = 0.93	β female allocation	0.82 (0.09)	***	-0.072 (0.13)	n.s.	0.41 (0.08)	***
	β male allocation	-0.17 (0.1)	**.**	0.28 (0.15)	**.**	0.033 (0.09)	n.s.
	γ female allocation	0.7 (0.2)	**	0.008 (0.32)	n.s.	0.38 (0.19)	*
	γ male allocation	0.3 (0.15)	*	-0.28 (0.23)	n.s.	0.04 (0.14)	n.s.
	γ female allocation, male allocation	0.25 (0.21)	n.s.	-0.18 (0.32)	n.s.	0.056 (0.19)	n.s.

Appendix S5. Estimated standardized linear (β_i), quadratic (γ_{ii}), and correlational (γ_{ij}) selection gradients for pistil (p) and stamen (st) number via female, male, and total reproductive success (RS) at the floral level from generalized additive models (*gam*). Standard errors and *P* values are obtained from bootstrapping procedures (see materials and methods for details).

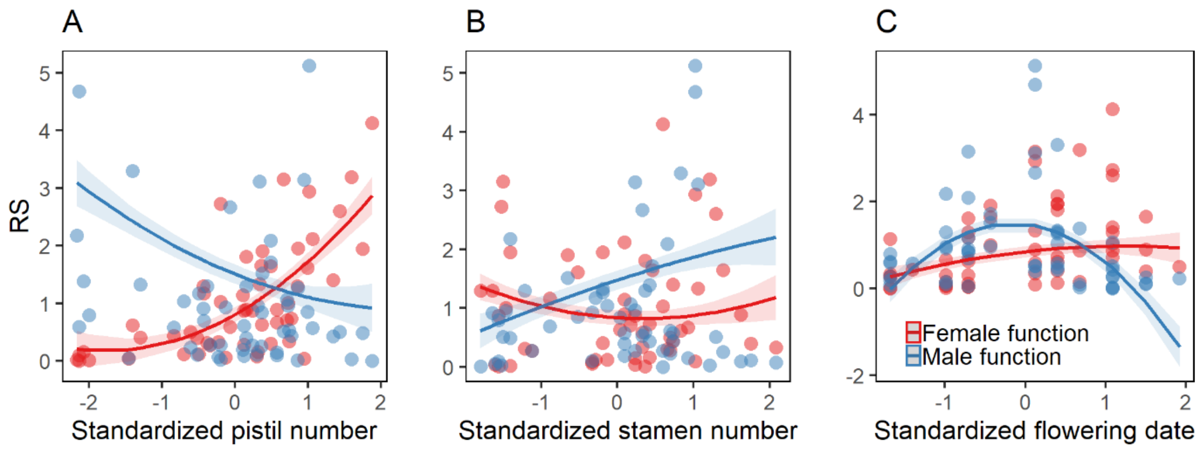
Notes: n.s. $P > 0.05$, $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$

		Female RS		**Male RS**		**Total RS**	
Inbreeding depression scenario	**Selection gradient**	**Estimate (SE)**	***P* value**	**Estimate (SE)**	***P* value**	**Estimate (SE)**	***P* value**
ID = 0	β_p	0.57 (0.03)	***	0.0098 (0.05)	n.s.	0.34 (0.02)	***
	β_{st}	-0.10 (0.02)	***	0.27 (0.03)	***	0.010 (0.02)	n.s.
	γ_p	-5.26 (0.96)	***	-15.17 (1.40)	***	-4.64 (0.52)	***
	γ_{st}	-1.45 (0.29)	***	-2.44 (0.35)	***	-0.96 (0.15)	***
	$\gamma_{p, st}$	-0.42 (0.15)	n.s.	-0.38 (0.21)	n.s.	-0.15 (0.08)	n.s.
ID = 0.93	β_p	0.79 (0.04)	***	-0.23 (0.1)	***	0.36 (0.03)	***
	β_{st}	-0.11 (0.03)	***	0.36 (0.05)	***	0.04 (0.04)	*
	γ_p	-2.69 (0.99)	***	-37.49 (3.03)	***	-6.29 (0.78)	***
	γ_{st}	-1.58 (0.28)	***	-7.57 (0.83)	***	-1.87 (0.22)	***
	$\gamma_{p, st}$	-0.3 (0.17)	n.s.	-0.05 (0.4)	n.s.	-0.32 (0.1)	**

Appendix S6. Linear (β) and quadratic (γ) selection gradients on five floral traits via female and male reproductive success (RS) in H flowers under the scenario of an inbreeding depression of 0.93. Selection gradients with a significant and marginally non-significant *P* value were in bold and italic, respectively. Differences in the selection gradients between female and male functions were tested by setting an interaction term between sex function and each trait in the multivariate generalized least square models (*gls*). Selection gradients were significantly different via the two sex functions on pistil number, stamen number, and flowering date (see Appendix S7 for visualization of the selection gradients).

Notes: **.** $P < 0.1$, $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$

		***d* = 0.93**		
Traits	**Selection gradients**	**Female RS (SE)**	**Male RS (SE)**	**Difference in selection**
Pistil number	β	**0.72 (0.15)*** **	**-0.51 (0.19)** **	***
	γ	**0.41 (0.19) ***	0.21 (0.24)	
Stamen number	β	-0.08 (0.11)	**0.42 (0.15)** **	**
	γ	0.24 (0.2)	-0.07 (0.26)	
Flowering date	β	*0.21 (0.12)* **.**	-0.21 (0.16)	*
	γ	-0.17 (0.25)	**-1.32 (0.31)*** **	**
Stalk height	β	-0.07 (0.14)	0.27 (0.18)	**.**
	γ	-0.05 (0.19)	0.03 (0.24)	
Petal length	β	-0.04 (0.12)	0.06 (0.15)	
	γ	-0.07 (0.12)	0.11 (0.15)	



Appendix S7. Difference in phenotypic selection gradients on pistil number, stamen number, and flowering date via female and male reproductive success in H flowers when *d* = 0.93. Each flower is represented by two points for its female (red) and male (blue) reproductive success (*N* = 58 flowers). The shaded ribbon indicates the standard error of the regression lines. See Appendix S6 for the values of selection gradients of each trait via the two sex functions.

CHAPTER III:

Disruptive selection via pollinators and seed predators on the height of flowers on a wind-dispersed alpine herb

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Abstract

Floral stalk height is known to affect pollination and seed dispersal of wind-dispersed grassland species, but it may also affect the attractiveness of flowers and fruits of animal-pollinated and animal-dispersed plants. Stalk height may thus be responsive to selection via interactions with both mutualist pollinators and seed dispersers, but also antagonist florivores and seed predators. In this study, we aimed to determine the effect of pollinators and seed predators on selection on floral stalk height in the insect-pollinated and wind-dispersed, alpine, andromonoecious herb *Pulsatilla alpina*, whose flowers also vary in their sex allocation and thus in the resources available to both mutualists and antagonists. We measured the resource status of individuals in terms of their size and the height of the vegetation surrounding plants of *P. alpina* at 11 sites. In one population, we recorded floral stalk height over an entire growing season and investigated its association with floral morphology and floral sex allocation (pistil and stamen number) and used leaf-removal manipulations to assess the effect of herbivory on floral stalk height. Finally, in four populations, we quantified phenotypic selection on floral stalk height in four female components of reproductive success before seed dispersal. Stalk height was positively associated with female allocation of the respective flower, the resource status of the individual, and the height of the surrounding vegetation, and negatively affected by leaf removal. Our results point to disruptive selection on stalk height in terms of both selection differentials and selection gradients for fertilization, seed predation, and seed maturation rates and to positive selection on stalk height in terms of a selection differential for mature seed number. Stalk height of *P. alpina* is a costly trait that affects female reproductive success via interactions with both mutualists and antagonists. We discuss the interplay between the resource status and selection imposed on female reproductive success and its likely role in the evolution of sex-allocation strategies, especially andromonoecy.

Keywords: Andromonoecy, Floral display, Floral evolution, *Phytomyza*, Ranunculaceae, Selection differential, Selection gradient, Sex allocation

Introduction

A central goal of plant evolutionary biology is to understand how selection acts on phenotypic traits in wild populations. The functional significance of variation in floral traits has attracted particularly keen attention (Caruso et al., 2019), not least because they vary so strikingly among species, populations, and individuals, but also because they can often be linked directly to key components of reproductive success and thus fitness (Gómez and Zamora, 2000; Maad, 2000; Ågren et al., 2013; Sletvold et al., 2013). For instance, the rate at which ovules are fertilized by self- versus outcross pollen (which affects the female component of reproductive success) may depend on factors such as the attractiveness of inflorescences and flowers (Waser, 1983; Caruso et al., 2019) and the manner in which flowers manipulate pollinator behavior (Schiestl and Johnson, 2013). Similarly, the ability of plants to disperse their pollen effectively to other flowers in the population (affecting male reproductive success) also depends on inflorescence and floral traits (Conner et al., 1996; Hodgins and Barrett, 2008). While interactions with mutualists thus likely play an important role in shaping floral evolution, floral traits may also influence fitness via antagonistic interactions with herbivores, florivores, and seed predators, through both their male and female components of fitness(Strauss and Whittall, 2006). For instance, both pollinators and antagonists have been shown to impose selection in the same or in different directions on floral color (Frey, 2007; Carlson and Holsinger, 2010; Ehrlén et al., 2012), floral size (Gómez, 2003; Pérez-Barrales et al., 2013), floral scent (Schiestl et al., 2011; Knauer and Schiestl, 2017), and nectar production (Kessler et al., 2015; Parachnowitsch et al., 2019). However, much less is known about how selection operates on traits that could be regarded as ancillary to flowering and floral function.

The height of the vegetative stalk on which the flowers and fruits develop is an ancillary trait that might affect plant fitness in several ways, positively or negatively (reviewed by Harder and Prusinkiewicz, 2013). Positive selection, through male fitness favoring longer branches or inflorescence stalks, has been shown for wind-pollinated species (Tonnabel et al., 2019), and a positive correlation between stalk height and pollinator visitation rate, which may strongly affect siring success, has been found in many animal-pollinated species (Galen, 1989; O'Connell and Johnston, 1998; Gómez, 2003; Sletvold and Ågren, 2015; Diniz et al., 2019). In species with wind-dispersed seeds, floral stalk height can also have a strong positive effect on seed-dispersal distances. For example, Greene and Johnson (1989) and Soons et al. (2004) showed that horizontal wind velocity and seed release height (i.e., stalk height) affected seed dispersal. Stalk height may also negatively affect a plant's female component of fitness if flowers on tall stalks are more easily seen or accessed by herbivores and seed predators, as has been found, for example, in *Castilleja linariaefolia* (Cariveau et al., 2004), *Erysimum mediohispanicum* (Gómez, 2003), and *Primula farinosa* (Ågren et al., 2013).

Selection on floral stalk height via its effect on both pollen and seed dispersers and potential antagonists also needs to be considered in the context of the marginal costs of stalk production under different resource levels and in the context of different vegetation types. First, to the extent that stalk height affects fitness positively, we should expect larger plants with more resources to produce taller stalks. In contrast, plants that have lost carbon and nutrients to prior herbivory, for instance, may be constrained to produce shorter stalks (Waite and Hutchings, 1982; Weiner, 2004), with potentially negative consequences for pollen and/or seed dispersal (Donohue, 1999; Sletvold et al., 2013). Second, as with overall plant height itself, the fitness implications of stalk height are likely to depend on the height of the surrounding vegetation, with plants producing taller stalks to maintain height above neighboring plants (Sletvold et al., 2013). Thus, while stalk height is an apparently simple quantitative trait, its expression is likely to be the outcome of responses to selection on norms of reaction to a plant's resource status (e.g., its overall size and history of herbivory), the height and density of vegetation in which it is expressed, and the extent to which different flowers vary in their sex allocation.

Optimal stalk height may differ for flowers with different sex allocation (i.e., the proportion of their resources committed to seed versus pollen production) if greater height benefits fitness via interactions with mutualists and antagonists more through one sex than the other. In many species, sex allocation varies considerably at both the individual and inflorescence or floral levels, and we might expect stalk height and sex allocation to covary in such species. For instance, Pickup and Barrett (2012) found that the height of stalks of males of wind-pollinated, dioecious *Rumex hastatulus* was greater than that of females during flowering, but that female stalk height was greater during fruiting and seed dispersal. To our knowledge, however, differential selection of stalk height as a function of sex allocation has hitherto not been investigated in any hermaphroditic or monoecious species. Furthermore, sex allocation is expected to vary as a function of plant size in many hermaphroditic species (de Jong and Klinkhamer, 1989; Klinkhamer et al., 1997), not only because larger plants have more resources and can thus potentially allocate more to the more costly sex (a so-called “budget effect” of size), but also because taller plants are better able to disperse seeds and/or pollen (a direct effect of plant size) (Klinkhamer et al., 1997).

Here, we explore the effects of floral stalk height on female components of plant reproductive success in the insect-pollinated and wind-dispersed alpine perennial herb *Pulsatilla alpina* (Ranunculaceae). This species displays wide variation among individuals not only in floral stalk height but also in floral sex allocation (the number of stamens and pistils per flower), plant size (and thus resource status), and the impact on resource status by herbivores. Flowers of *P. alpina* are presented to pollinators early in the growing season, as soon as snows melt. They are pollinated largely by generalist dipteran pollinators (Appendix S1), which might be attracted to flowers on taller stalks. However, they are also visited by a specialist dipteran seed predator that lays its eggs in the gynoecium and its larvae eat the seeds (technically the achenes), potentially diminishing the benefits of greater height during flowering. In addition, stalk height is likely to have a positive effect on seed dispersal by wind, particularly when plants are growing in tall vegetation.

Here, we first describe variation in floral stalk height and other floral characters within and among populations of *P. alpina* in the Swiss Alps. We then assess phenotypic selection on the female components of reproductive success in terms of morphological variation in flowers and floral stalks. In particular, we address the following questions:

(1) How does stalk height vary among populations, and, in particular, as a function of vegetation height? We expected plants in taller vegetation to have taller stalks.
(2) Within a population, how does stalk height vary across developmental stages, and how does its development correspond to that of other floral traits? To the extent that taller stalks during fruiting and seed dispersal have a positive influence on plant fitness, we expected stalk height to increase between flowering and fruiting in this hermaphroditic species, as found previously for females of a dioecious herb (Pickup and Barrett, 2012).
(3) Does stalk height covary with floral sex allocation? A finding of greater stalk height for flowers with greater relative allocation to one sex would be consistent with fitness through that sex benefitting more from floral height than through the other.
(4) To what extent might herbivory on leaves and vegetative shoots impact the height of floral stalks plants are able to produce? If producing tall stalks is costly, we expected simulated herbivory to cause plants to produce shorter stalks.

(5) How does stalk height influence the ovule fertilization rate and the seed predation rate? If both of these rates are greater in flowers on taller stalks, then different components of selection via pollinators and seed predators operate in different directions.

Materials and methods

Study species

Pulsatilla alpina (L.) Delarbre (Ranunculaceae) is a perennial hemicryptophyte distributed from subalpine to alpine grassland in central Europe (Lauber et al., 2018). Several shoots emerge from a perennial rhizome soon after the snowmelt, from early May to July; these shoots may be vegetative or reproductive. The plants vary greatly in both aboveground size (number of leaves and flowers) and in the size of the persistent underground rhizome. Aboveground herbivory in the study populations is mainly the result of direct consumption or trampling by cattle, usually in late summer.

A single flower with white, showy tepals is produced at the apex of its floral shoot. Individuals produce both male and hermaphroditic flowers (the species is thus andromonoecious), with wide variation in their number of pistils and stamens per flower; i.e., hermaphroditic flowers vary quantitatively in their sex allocation. Phenotypically, male flowers bear no pistil, and the pistil number in hermaphroditic flowers varies from about 10 to about 400. Each pistil contains only one ovule. Stamen number varies from about 150 to about 400 in both male and hermaphroditic flowers. Flower number varies from one to 20 flowers among individuals and populations. Fruits ripen to produce achenes with an elongated pappus that promotes dispersal by wind (Muller-Schneider, 1986; Vittoz and Engler, 2007). After achenes are dispersed in autumn, the aboveground parts of the plants wither, but individuals persist underground as a rhizome until the next spring.

Flowers of *P. alpina* are visited by both dipteran pollinators and seed predators. The main pollinators of *P. alpina* are house flies and syrphids (Appendix S1), which visit the flowers for pollen (Szentpéteri et al., 2008). Flowers of *P. alpina* are also visited by *Phytomyza* species, which are monophagous, dipteran seed predators. These flies mate in the flower, the female adults oviposit on the pistils, and the larvae eat the pollinated ovules during the fruiting stage, as found for other *Phytomyza* species that eat seeds within the achenes (Winkler et al., 2009).

Survey of variation in stalk height among populations

We conducted our survey and experiments in 13 populations in the pre-Alps of the Canton Vaud, Switzerland, over three consecutive years from 2019 to 2021 (see Appendix S2 for the details of each population). To characterize variation in stalk height among populations, we sampled 11 populations at the end of the growing season. In each population, we established four to five transects across the population and permanently marked them. Within each 1-m-wide transect, we sampled the tallest floral stalk at the fruiting stage from around 20 individuals of *P. alpina*; i.e., we sampled a total of approximately 80 to 100 individuals per population, though in populations with a high proportion of nonflowering individuals or low population density (i.e., populations S1+, S4, and LS2), only around 50 flowering individuals were sampled. In all, we sampled stalks from 757 individuals from 41 transects at the end of the growing season. We measured the stalk height as the distance between the bottom of the stalk aboveground and the receptacle of the flower to which the achenes are attached.

To determine the effect of intrinsic and extrinsic factors on the variation of stalk height among populations, we recorded the total number of flowers on each sampled individual and height classes for the vegetation along the transects in the early and late growing season. We used the total flower number to infer the plant’s resource status (larger plants produce more flowers). We measured vegetation height in a semiquantitative way by assigning the vegetation along the transects to six height categories, i.e., 0 cm, 1–10 cm, 11–30 cm, 31–60 cm, 61–100 cm, and >100 cm, and recorded the cover of each height class on a six-interval scale: <1%, 1%–5%, 5%–25%, 25%–50%, 50%–75%, and 75%–100%. We calculated the mean height for each transect by weighting height classes in terms of the centroid of their cover class for the transect.

Variation in stalk height through developmental stages and correlation with other floral traits at the flower level

To characterize variation in stalk height at the flower level, we followed the development of floral stalks in 60 flowers from 20 individuals that varied in plant size and flower number (from one to 13 flowers) through the growing season in population S1. We recorded each floral stalk every 3–5 days, from the budding stage toward the end of the flowering stage, and every 7–20 days until the end of the fruiting stage. We defined the period of the budding stage as beginning with the emergence of the floral stalk and ending when the flower opened its tepals. The flowering stage was defined as beginning when flowers opened and ending when they wilted. The fruiting stage began as flowers wilted and ended with the commencement of fruit dispersal.

To evaluate the correlation of flower height with other important floral traits of the species, we recorded tepal length, pistil number, and stamen number at the end of the flowering stage. On dry days when tepals were fully expanded, we measured the length of the largest tepal of the flowers, which is at the outer whorl in most of the cases, from the tip of the tepal toward its bottom attached to the receptacle. To quantify sex allocation of each flower because the species possesses a great variation in the number of pistils and stamens within a flower, we photographed the flowers and counted the pistil number from top-view photographs and the stamen number from side-view photographs, then multiplied by two.

The effect of leaf removal on floral stalk height

To manipulate the potential resource availability of individuals for investment toward reproduction, we conducted a leaf-removal experiment in two populations (LL3, 2019, N = 101; S1, 2020, N = 72) in which herbivory was deemed to be low, based on personal observations in 2018. Around 12 flowering individuals bearing one, two, or more than two flowers were arbitrarily chosen and labeled with a metal tag on the ground at the beginning of the flowering season within each subplot in each population and were randomly assigned to either a leaf-removal (LR) or a control (C) treatment, by a random draw of lots. For the LR treatment, two thirds of the total leaves were removed at the beginning of the flowering season. A second round of leaf removal was conducted a month later to ensure that all the newly emerged leaf stalks were defoliated. Control plants were not damaged. In the next flowering season, each flower was labeled with a tag and photographed. We measured the height of all the floral stalks of the individuals at the end of the flowering stage as described in the previous section.

Selection differentials and selection gradients on stalk height for female fitness components before seed dispersal

To evaluate the female component of selection on floral stalk height, we quantified achene fate for 322 seed families collected in four populations in 2019, i.e., population S1 (N = 95 flowers from

45 individuals), population S2 (N = 74 flowers from 42 individuals), population LL1 (N = 62 flowers from 42 individuals), and population LL4 (N = 91 flowers from 41 individuals). We marked the flowers with a tag at the beginning of the flowering season, chosen arbitrarily from different individuals varying in their size. We measured the same floral traits at the end of the flowering stage, as described in the previous section, i.e., stalk height, tepal length, and the number of stamens. We calculated the number of pistils as the sum of the number in each of the three achene categories in each flower (see below for details). We collected the achenes of the labeled flowers at the end of the growing season before they started to disperse. Because male flowers bore no achene, we used only hermaphroditic flowers in the analysis.

To quantify the predispersal components of female reproductive success, we separated achenes into three categories: unfertilized, predated, and mature, based on morphological assessment (see Appendix S3 for a detailed description). We interpreted the seed fertilization rate as a reflection of pollinator visitation, autonomous selfing, and resource limitation (see Discussion). The seed predation rate reflects selection imposed mainly by the *Phytomyza* seed predator, but may also be affected by selection via organisms at higher trophic levels, e.g., parasitoid wasps, which parasitize the seed predators and thus potentially reduce the seed predation rate. The seed maturation rate describes the proportion of all achenes that were fertilized and not predated. Lastly, we used mature achene number as one of the fitness components reflecting the contribution to the gene pool of the next generation via the female function. In summary, we considered four fitness components in our analyses: the number of mature achenes, the fertilization rate (the sum of predated and mature achenes divided by all achenes produced in the flower), the achene predation rate (the number of predated achenes divided by the number of fertilized achenes), and achene maturation rate (the number of mature achenes divided by all achenes produced in the flower). For greater clarity in interpreting directions of selection, in what follows, we refer to the rate of nonpredation (calculated as 1 minus predation rate) instead of the predation rate.

Statistical analyses

We conducted all analyses within the R statistical framework v 4.0.3 (R Core Team, 2021) using package lme4 for (generalized) linear mixed models (Bates et al., 2015). We evaluated the fit of each model with the R package DHARMa (Hartig, 2019).

We used a linear mixed model to evaluate the dependence of stalk height on flower number and vegetation height during the early and the late season, with population, flower number, and average vegetation height during the early and the late season as fixed effects. We considered transects as a random effect that grouped different individuals. To evaluate the relationship among stalk height, tepal length, pistil number, and stamen number, we calculated the Pearson correlation for each trait pair. To evaluate the effect of leaf removal on stalk height in the next season, we used a linear mixed model, with population, treatment, and their interaction as fixed effects. We considered individual as a random effect to account for the fact that more than one stalk was sampled from some individuals.

To evaluate the phenotypic selection differentials and selection gradients on stalk height for the four components of female reproductive success, we used generalized linear mixed models for fertilization rate, nonpredation rate, and seed maturation rate and a linear mixed model for relative female fitness. The difference between a selection differential and a selection gradient is that the former measures the total strength of selection on the trait (direct and indirect effect via correlation with other traits), while the latter measures only the strength of direct selection (partial effect) on the trait (Brodie et al., 1995). We calculated the relative mature achene number by dividing the number of mature achenes of each flower by the mean number of mature achenes across the population. We standardized stalk height, tepal length, pistil number, and stamen number for each population to a mean of zero and a standard deviation of one.

To determine the linear (S) and quadratic (C_{ii}) selection differentials, which are the total strength of selection on the trait (direct and indirect), we set linear and quadratic terms for stalk height as fixed effects and considered the interactions with the population in each simple regression model (Lande and Arnold, 1983; Matsumura et al., 2012). We used generalized linear mixed models with a logistic function for binomial response variables, i.e., fertilization rate, nonpredation rate, and seed maturation rate, and a linear mixed model for relative mature achene number. We used the emtrends function in the R package emmeans to extract the regression coefficients and standard errors of the general effects and each population from the models (Lenth, 2020). Regression coefficients and their standard errors from the generalized models of each population were adjusted by multiplying the value with a constant to approximate the selection differentials (Janzen and Stern, 1998). The constant is the average of $W(z)[1 - W(z)]$, where $W(z)$ is the predicted fitness value for each individual in the studied population using the estimated logistic regression coefficients. A significant linear differential indicates a directional selection on the trait, and a significant quadratic differential in conjunction with a local maximum and minimum indicates stabilizing or disruptive selection, respectively. A significant interaction between a floral trait and the population indicates a difference in selection among populations. For all quadratic differentials, we multiplied the regression coefficient by two to obtain the correct estimate of stabilizing or disruptive selection (Stinchcombe et al., 2008). We set individual identity as a random effect to account for the fact that more than one flower was sampled in some individuals in all the regression models. In addition, we set flower identity as a random effect in the generalized models to account for the fact that the unit of the binomial response variables (e.g., fertilization rate) is one achene grouped with the other achenes of the same flower.

To determine the linear (β) and quadratic (γ_{ii}) selection gradients, we set linear and quadratic terms of the four floral traits as fixed effects and considered the interactions with population in each multiple regression model (Lande and Arnold, 1983; Morrissey, 2014). This approach allowed us to extract the partial effect on the fitness components by each trait. We used generalized linear mixed models with a logistic function for binomial fitness components and a linear mixed model for relative mature achene number. We set random effects as described above for the simple regression models. Regression coefficients and their standard error from the generalized models were adjusted by multiplying the value with a constant, as described above (Janzen and Stern, 1998). For all quadratic gradients, we multiplied the regression coefficients by two to obtain the correct estimate of stabilizing or disruptive selection (Stinchcombe et al., 2008).

Results

Among-population variation in floral stalk height

Across all individuals sampled, floral stalk height varied from 21 to 72 cm (mean ± SD: 46.5 ± 8.71), with substantial and significant ($P < 0.001$) variation among populations, from a minimum in population S4 (34.9 ± 6.84 cm) to a maximum in population LM1 (52.9 ± 9.28 cm) (Figure 1A). Individuals producing more flowers also produced taller stalks ($P < 0.001$) (Figure 1B). Individuals in taller vegetation at late season produced taller stalks ($P < 0.01$) (Figure 1C), but not at early season (Appendix S4). These patterns were consistent within and among populations (Figure 1B, C).

Variation in floral stalk height over time and its correlation with other floral traits

We fitted a local polynomial regression on stalk height through developmental stages to capture the dynamics of stalk height (Figure 2A). Stalks continued to elongate during the budding and flowering stage, roughly the first 20 days after their emergence aboveground, but showed no further elongation after the end of flowering. Stalk height correlated positively with pistil number ($r = 0.65$, $P < 0.001$) and tepal length ($r = 0.51$, $P < 0.001$) (Figure 2A, B). The stalk height for male and hermaphroditic flowers was 30.4 ± 6.69 and 49.9 ± 6.96 cm, respectively, at the end of the male stage, and was 32.8 ± 6.18 and 55.8 ± 10.2 cm, respectively, at the end of the fruiting stage.

Effects of leaf removal on floral stalk height

Plants subject to leaf removal produced significantly shorter stalks the following season in two populations than did control plants (LR: 33.7 ± 9.04, C: 39.4 ± 12.4 cm; $P < 0.01$). The interaction between population and treatment was not significant (Figure 3; Appendix S5).

Selection differential on floral stalk height for predispersal components of female reproductive success

In total, 67,798 achenes were counted to determine the rate of fertilization, the rate of nonpredation, the rate of seed maturation, and the number of mature seeds. The mean and standard deviation of the fitness components and stalk height of the four populations can be found in Appendix S6.

Overall, our models found largely consistent trends for the selection differentials on stalk height across the studied populations in the four fitness components (Table 1, Figure 4). There was a tendency toward positive quadratic effects of stalk height on fertilization rate (general linear coefficient = 0.0722, $P > 0.05$; quadratic coefficient = 0.109, $P < 0.01$), rate of nonpredation (general linear coefficient = –0.121, $P > 0.05$; quadratic coefficient = 0.119, $P < 0.05$), and seed maturation rate (general linear coefficient = 0.006, $P > 0.05$; quadratic coefficient = 0.133, $P < 0.01$). There was a tendency toward a positive linear effect of stalk height on relative mature seed number across the four populations (general linear coefficient = 0.186, $P < 0.001$; quadratic: $\gamma = 0.004$, $P > 0.05$) (See Table 1 and Figure 4 for the values and illustrations of the selection differentials of each population and Appendix S7 for figures showing the general effects). The interaction with population was nonsignificant for both linear and quadratic terms for all the fitness components, with a marginally nonsignificant interaction between population and the quadratic terms for the rate of nonpredation (Figure 4B; $P = 0.064$). There was a single minimum value for the fertilization and seed maturation rates within the phenotypic range of stalk height in each of the four populations predicted by the models, indicating a largely consistent pattern of a disruptive selection on stalk height for the two fitness components (Figure 4). For the rate of nonpredation, a single minimum value was found in three populations, and a maximum was found in one population (Figure 4), pointing to disruptive selection in the former three and stabilizing selection in the latter. The pattern of disruptive selection on stalk height in the fertilization rate, the rate of nonpredation, and the seed maturation rate may have been driven to some extent by the influence of extreme phenotypes, as evidence for disruptive selection was substantially weakened when extreme phenotypes were removed from the analysis (see Appendix S8). Lastly, a maximum value of relative mature seed number was found at the positive end of the range of standardized stalk height in three populations, pointing to positive directional selection (Figure 4).

Selection gradients on floral stalk height for predispersal components of female reproductive success

We focused on the main results of the selection gradient on floral stalk height in the four female fitness components from the multiple regression models. A complete table of the results of the multiple regression models for all traits can be found in Appendix S9.

Overall, the selection gradients on stalk height were largely consistent across the studied populations for the four fitness components, as also found for the selection differentials (Table 1). There was a tendency toward positive quadratic effects for the regression of stalk height on fertilization rate (linear coefficient = 0.045, $P > 0.05$; quadratic coefficient = 0.102, $P < 0.05$), rate of nonpredation (linear coefficient = 0.01, $P > 0.05$; quadratic coefficient = 0.083, $P < 0.05$), and seed maturation rate (linear coefficient = 0.028, $P > 0.05$; quadratic coefficient = 0.115, $P < 0.01$). Neither linear nor quadratic terms were significant for regressions of stalk height on relative mature seed number (linear coefficient = 0.01, $P > 0.05$; quadratic coefficient = 0.03, $P > 0.05$) (Table 1; Appendix S9). The interaction with population was nonsignificant in both linear and quadratic terms for fertilization rate, seed maturation rate, and relative female fitness, while the interaction with population fell just short of significance for the rate of nonpredation (P = 0.081 and 0.082 for linear and quadratic, respectively) (Appendix S9).

The direction and shape of selection differentials and selection gradients of each population were largely consistent, though the pattern was statistically significant for only some of them, likely due to a variation in the strength of selection, or due to low statistical power. Nonetheless, the consistency of the effects of stalk height on the four fitness components among populations was reflected in highly significant values for the general pattern (Appendices S7 and S9).

Discussion

Our study provides evidence for unusual disruptive selection on floral stalk height in *P. alpina* in terms of three components of reproductive success. Our results also indicate that floral stalks are costly to produce and that investment in such stalks should likely be attributed largely to female rather than male components of reproductive success, a feature that may help to explain the andromonoecious sexual system of this perennial herb.

Disruptive selection on stalk height for three components of female reproductive success

Our results point to disruptive selection on floral stalk height in *P. alpina*, with flowers on taller and shorter stalks having a higher fertilization rate, lower seed predation rate, and higher seed maturation rate than those of intermediate height. It is noteworthy that we found a positive selection differential but not a positive selection gradient for relative mature seed number across the populations sampled (Table 1, Figure 4; Appendices S7, S9). Given that the selection differential measures the total strength of selection on stalk height (direct and indirect effect via correlation with other traits) and the selection gradient measures only the strength of direct selection (partial effect) on stalk height (Brodie et al., 1995), it is possible that the higher relative mature seed number associated with taller stalks (i.e., positive selection differential) is an indirect effect through a positive correlation with, for instance, pistil number.

The disruptive selection on stalk height in terms of fertilization rate (Table 1, Figure 4A; Appendix S7A) may be a result of both pollinator preference and facilitated or autonomous selfing. On the one hand, several previous studies have found a positive selection on stalk height by the pollinators on female fitness (Galen, 1989; O'Connell and Johnston, 1998; Sletvold et al., 2015), and a strong preference for flowers on higher stalks in the grassland plant community has been found in several syrphid fly species, which are the main pollinators of *P. alpina* (Klecka et al., 2018). On the other hand, *P. alpina* individuals produce actinomorphic flowers with pistils surrounded by numerous stamens, as is found in many basal eudicots. The pistils in the outer whorl that are directly beside the stamens may be subject to self-pollination, either autonomously or facilitated by pollinators. Because short stalks usually have fewer pistils, it is possible that the high fertilization rate in short stalks is a consequence of such selfing. If so, these two mechanisms may ultimately lead to the observed disruptive selection in the fertilization rate, with the degree of inbreeding depression determining the direction of the selection (e.g., Briscoe Runquist et al. 2017). Estimates of the selfing rate in flowers with different allocation strategies and stalk heights would be worthwhile to test this hypothesis.

On the other hand, our finding of disruptive selection on stalk height due to seed predation differs from that of most studies, which have tended to reveal directional negative selection on stalk height by antagonists, from invertebrates to ungulates (Gómez, 2003, 2008; Cariveau et al., 2004; Ehrlén et al., 2012; Ågren et al., 2013). However, these other studies have typically considered only directional selection in their analyses, i.e., only linear terms were included in the relevant statistical models analyzed and disruptive or stabilizing selection was not formally tested (e.g., Cariveau et al. 2004; Ehrlén et al. 2012). In one of the few studies that did consider quadratic terms in the multiple regression, Gómez (2008) found evidence for stabilizing selection on stalk height in the rate of seed predation in *Erysimum mediohispanicum*, in contrast to our finding of disruptive selection. Furthermore, it is likely that the seed predation rate in *P. alpina* depends not only on the preference of seed predators, but also on that of the predators or parasites such as parasitoids of the seed predators (personal observations), as in several other systems (Molau et al., 1989; Gómez and Zamora, 1994). However, the host-feeding strategies of parasitoids are complicated, and most studies have focused only on olfactory cues (Jervis and Kidd, 1986; Giunti et al., 2015). Thus, how parasitoids play a role in the selection of a trait such as stalk height remains largely unexplored.

Whatever its causes, the consistency among populations of the signatures of disruptive selection on floral stalk height is striking, not only through pollinators but also through seed predators. Some studies have reported counteracting selection by pollinators and seed predators (Gómez, 2007; Ehrlén et al., 2012; Thomann et al., 2018), while others found evidence for selection on a trait by one of the agents (Cariveau et al., 2004). Furthermore, some studies have shown a geographic mosaic (sensu Thompson 1999) in the direction and shape of selection imposed by different agents such as pollinators, herbivores, or seed predators (Gómez and Zamora, 2000; Gómez et al., 2009; Ågren et al., 2013). These studies thus contrast with our finding of a rather constant pattern across the four populations. It seems likely that stalk height in *P. alpina* varies not so much because of differences among populations in the strength and direction of selection due to mutualists or antagonists, but rather as a result of heterogeneity in, for example, the sex allocation of their flowers, competition with other plants in the surrounding vegetation, the resource status of the plants, and perhaps differences in selection between male and female components of fitness.

Female sex allocation, inferred cost of stalk height, and evolution of the sexual system

Our results suggest that stalk height affects the female component of reproductive success in *P. alpina* and may be less relevant to male reproductive success. First, we found that stalk height correlated positively with floral pistil number but not stamen number, suggesting that plants investing in taller stalks benefit more through their seeds than through their pollen. Second, and consistent with this view, the stalks of purely male flowers were shorter than hermaphroditic flowers at all development stages assessed. Third, the positive correlation we observed between stalk height and vegetation height during late but not early season is also consistent with the idea that taller stalks likely improve seed dispersal distance, especially in wind-dispersed species in grassland (Greene and Johnson, 1989; Soons et al., 2004). We found that larger plants produced taller stalks and that simulated herbivory caused plants to produce shorter stalks in the following season, suggesting that stalk growth represents a net drain on a plant's resources. In this sense, the cost of floral stalks in *P. alpina* should probably be considered largely as a component of female allocation. Our conjecture that floral stalk growth in *P. alpina* is the outcome of selection via female function more than via male function contrasts with the conclusions reached in studies of other species in which greater floral height more typically enhances pollen export (e.g., O'Connell and Johnston 1998; Maad 2000; Eppley and Pannell 2007) or even that stalk height is under negative selection via male fitness.

The differences in inferred selection via female versus male reproductive success in *P. alpina* may help to explain its andromonoecious floral strategy, whereby individuals produce both male and hermaphroditic flowers. Hypotheses for the evolution of andromonoecy have hitherto invoked selection via male reproductive success (Bertin, 1982; Spalik, 1991), with empirical studies asking whether male flowers increase pollinator attraction (Podolsky, 1992; Ehrlén, 1993), enhance pollen output (Elle and Meagher, 2000; Cuevas and Polito, 2004; Dai and Galloway, 2012), or reduce sexual interference within a flower (Quesada-Aguilar et al., 2008). These explanations are inadequate to explain andromonoecy in *P. alpina.* Rather, the association between female sex allocation and stalk height in *P. alpina* constitutes indirect support for an alternative idea, advanced by de Jong et al. (2008), that andromonoecy should be favored when the costs of the female function are higher than the costs of stamens and pollinator attraction. Producing both male and hermaphrodite flowers allows plants substantial flexibility to adjust their sex allocation to promote seed dispersal by wind while responding to constraints imposed by their resource status (Spalik, 1991; Zhang and Jiang, 2002): small plants or those with a resource pool compromised by herbivory or previously high investment in reproduction reduce their investment in female function (and the necessity to promote it with long stalks) by producing male flowers. Interestingly, the link between wind dispersal of seeds and andromonoecy has been found in other grassland species that also produce elongated stalks, such as species in the Apiaceae (Jury, 1996; Schlessman, 2010) and Liliaceae (Peruzzi, 2012; Zhang et al., 2014; Niu et al., 2017). It will be important to evaluate these conjectures for *P. alpina* with reference to estimates of both the male and the female components of reproductive success simultaneously.

Conclusions

Taken together, our results indicate that selection on the “simple” trait of floral stalk height is under the complex influence of both pollinators and seed predators in the context of variation in the sex allocation of the flowers, the height of local competitors in the surrounding vegetation, and the action of defoliating herbivores. The andromonoecious sexual system of *P. alpina* may thus profitably be viewed as a reaction norm of sex allocation at the flower and plant level to these complex interactions. Nevertheless, our study has focused on assessing fitness through only the female component of plant fitness, and a complete picture of selection on reaction norms of sex allocation and the expression of ancillary traits such as floral stalk height awaits a complimentary assessment of selection through male fitness, as well as the mating system, interactions between seed predators and their potential antagonists such as parasitoids, and interactions between genotypes and the environment.

Acknowledgments

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Table 1. Selection differentials and selection gradients on stalk height of four fitness components in four studied populations. Linear (*S*) and quadratic (C_{ii}) selection differentials of stalk height and the standard errors were derived from single regression models. Linear (β) and quadratic (γ_{ii}) selection gradients and the standard errors of four floral traits were derived from multiple regression models with the other floral traits. Significant values ($P < 0.05$) are in bold. Marginally nonsignificant values ($0.05 < P < 0.1$) are in italic.

	Fertilization rate				**Rate of nonpredation**				**Seed maturation rate**				**Relative mature seed number**			
Heading Population	**LL1**	**LL4**	**S1**	**S2**	**LL1**	**LL4**	**S1**	**S2**	**LL1**	**LL4**	**S1**	**S2**	**LL1**	**LL4**	**S1**	**S2**
S (Linear selection differential)	0.012 ±0.023	0.014 ±0.023	0.027 ±0.024	0.009 ±0.027	0.008 ±0.024	–0.04 ±0.026	–0.033 ±0.028	–0.034 ±0.03	0.008 ±0.022	–0.009 ±0.023	0.007 ±0.026	–0.002 ±0.026	*0.147* ±0.078	*0.135* ±0.073	**0.311** ±0.074	*0.152* ±0.085
C_{ii} (Quadratic selection differential)	*0.073* ±0.039	0.017 ±0.026	0.024 ±0.028	*0.071* ±0.038	**0.115** ±0.041	*0.059* ±0.032	0.026 ±0.032	–0.029 ±0.04	**0.101** ±0.038	0.034 ±0.026	0.034 ±0.03	0.025 ±0.035	0.175 ±0.133	0.036 ±0.081	–0.114 ±0.087	–0.063 ±0.105
β (Linear selection gradient)	–0.004 ±0.024	0.009 ±0.024	0.011 ±0.026	0.011 ±0.027	**0.048** ±0.024	–0.041 ±0.027	–0.018 ±0.028	0.007 ±0.032	0.014 ±0.023	–0.015 ±0.024	0 ±0.028	0.02 ±0.029	0.007 ±0.073	–0.038 ±0.071	0.095 ±0.077	–0.023 ±0.087
γ_{ii} (Quadratic selection gradient)	*0.067* ±0.039	0.01 ±0.025	0.02 ±0.03	**0.021** ±0.03	**0.086** ±0.038	*0.054* ±0.029	0.018 ±0.031	–0.041 ±0.038	**0.087** ±0.037	0.027 ±0.025	0.027 0.031	0.027 ±0.034	*0.214* ±0.12	0.023 ±0.072	-0.03 ±0.084	0.031 ±0.097

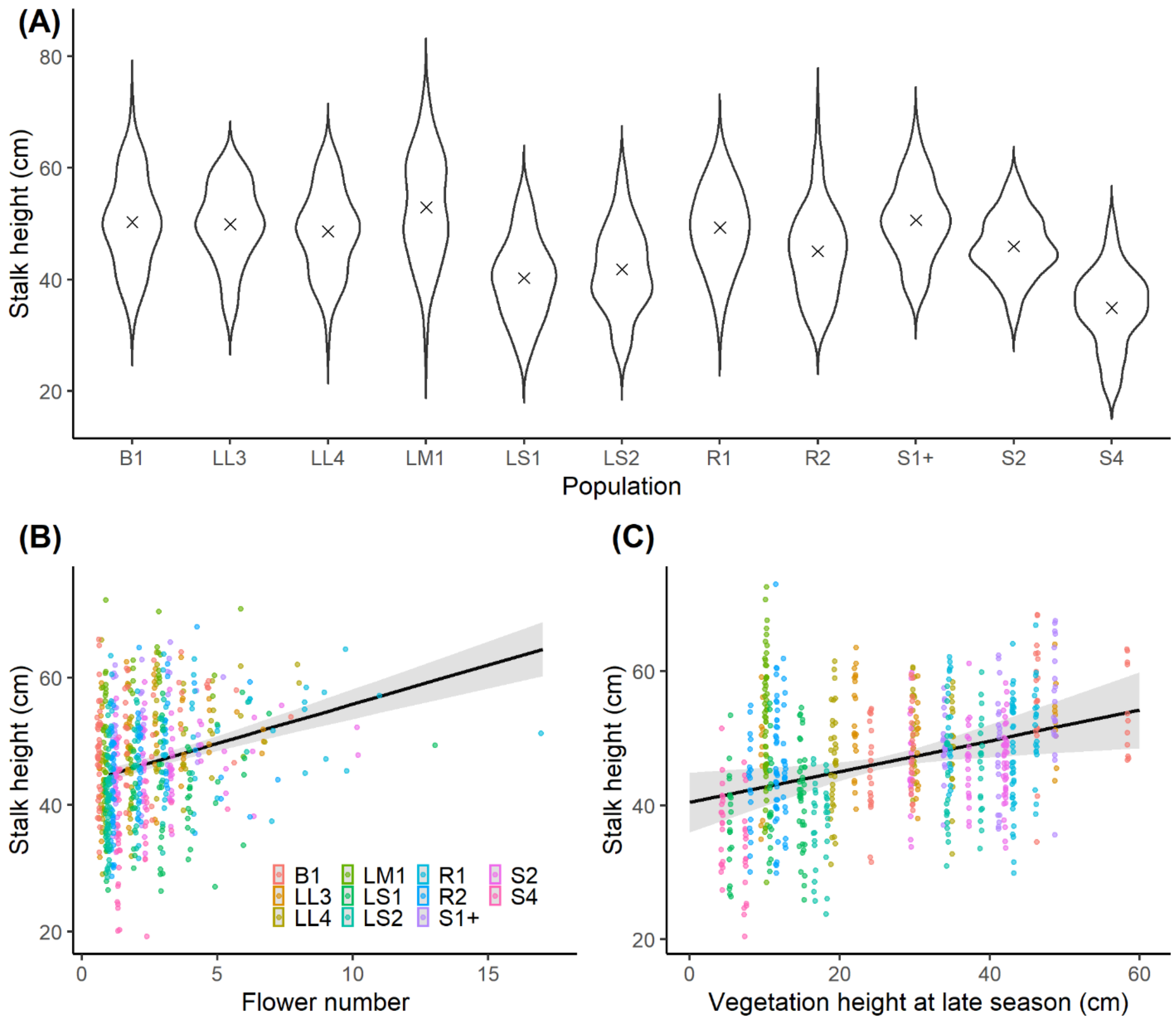


Figure 1. (A) Violin plot showing the variation in stalk height at the end of the growing season across 11 populations studied. The mean of each population is indicated by crosses. (B) Predicted values of stalk height as a function of flower number. (C) Predicted values of stalk height as a function of late-season vegetation height. Data points from different populations are shown in different colors in (B) and (C). The shaded ribbon indicates the 95% confidence interval of the regression lines in (B) and (C).

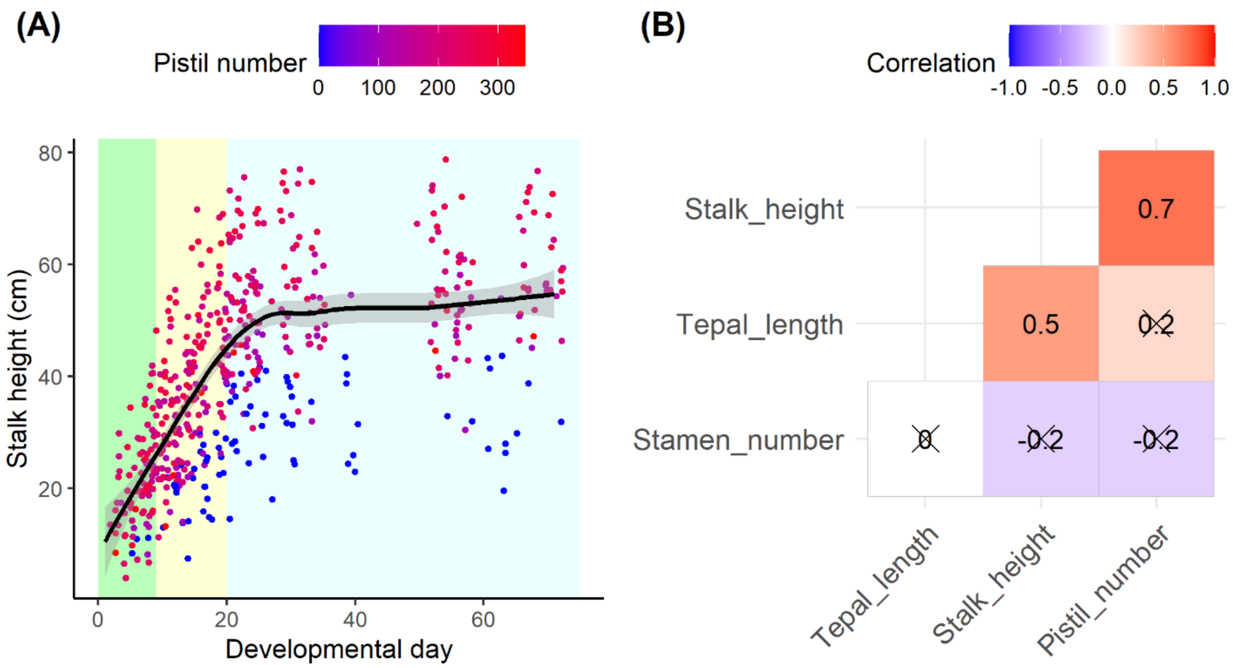


Figure 2. (A) Dynamics of the height of 60 floral stalks across the season from bud stage to fruiting stage. The color gradient of the points indicates the number of pistils of the flower. Light green: bud stage; yellow: flowering stage; blue: fruiting stage. The shaded ribbon indicates the 95% confidence interval of the smoothed lines. (B) Correlation matrix for stalk height, tepal length, pistil number, and stamen number. Floral traits were measured at the end of the flowering stage. Nonsignificant correlations (*P* > 0.05) are shown under a cross.

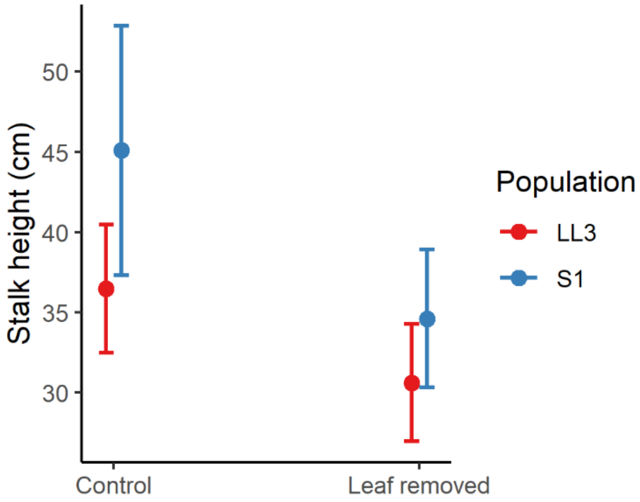


Figure 3. Predicted values of stalk height in year t after leaf removal (or not) in year $t - 1$. Results are shown for two populations (see inset legend). The upper and lower bar indicates the 95% confidence interval. Plants from the leaf removal treatment produced shorter stalks in the next season in both the two populations ($P < 0.01$).

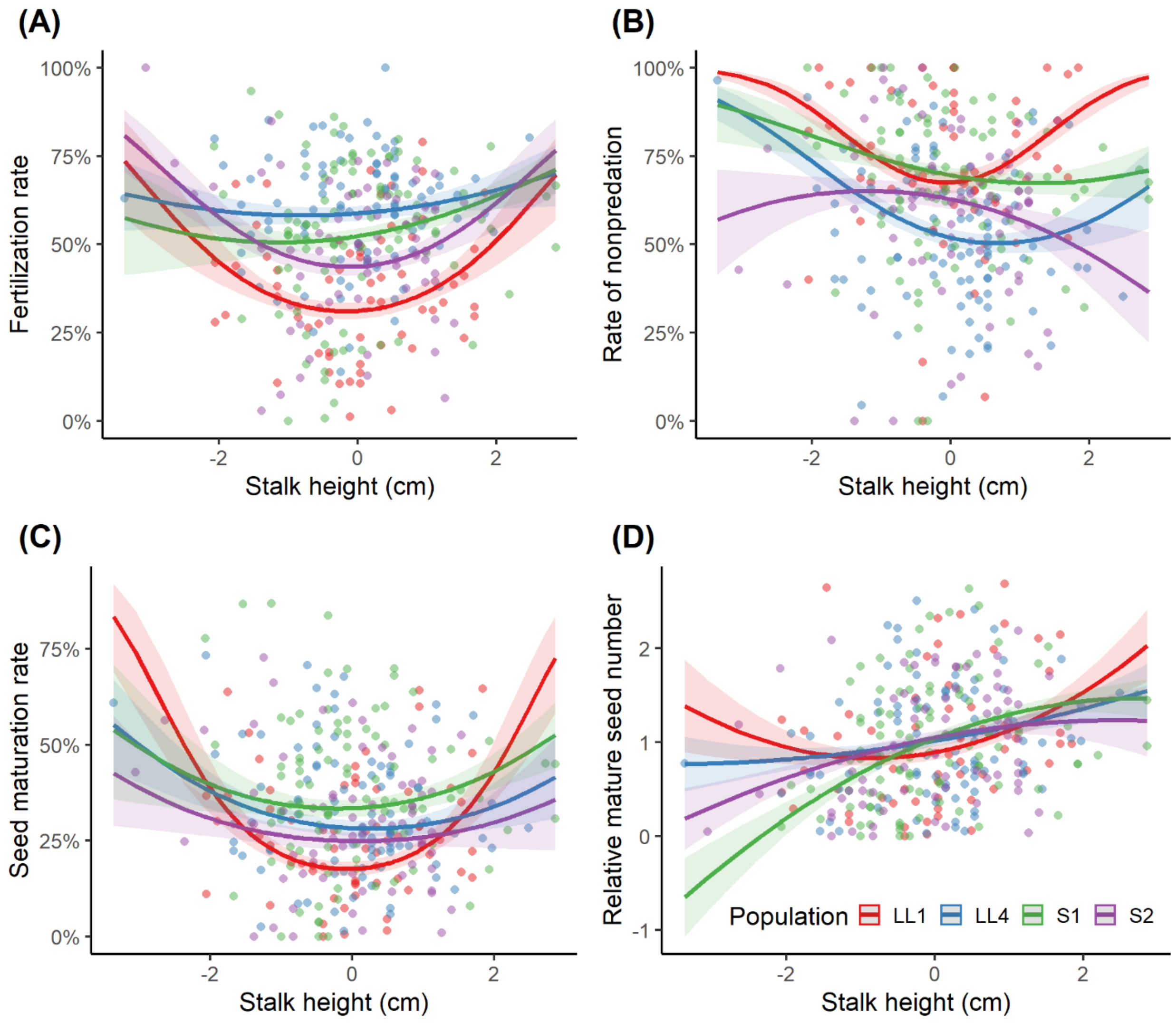
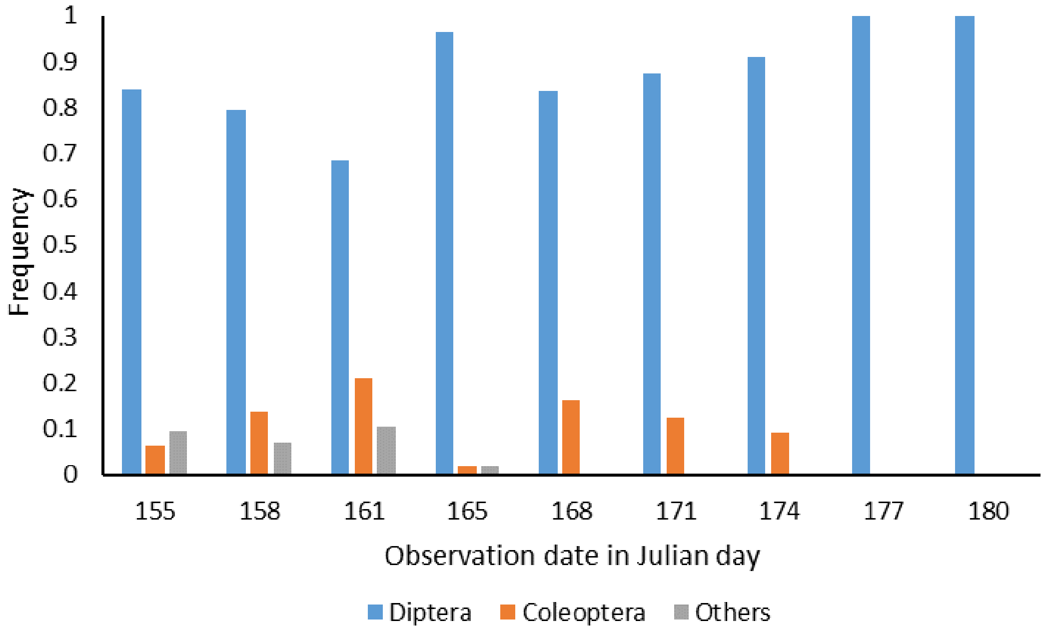


Figure 4. Visualization of the relationship between standardized stalk height at the end of the flowering stage and (A) fertilization rate, (B) nonpredation rate, (C) seed maturation rate, and (D) relative mature seed number among populations from single regression models evaluating the selection differentials. Raw data points and regression lines of the populations studied are shown in different colors (interactions between stalk height and population were all nonsignificant). The shaded ribbons indicate the standard errors of the regression lines. See Table 1 for the significant values of the linear and quadratic selection differentials

Supplementary information

Appendix S1. Pollinator assemblage of *P. alpina* in population LM in 2018. The frequency of different groups of insect visitors during the course of the flowering season. Three to five 10-minute observations were conducted on each observation day. All the insects observed visiting flowers were recorded. In total, 265 floral visitations were recorded. Hymenoptera, Lepidoptera, and Hemiptera visitors are shown in the category "Others". Diptera, including the house fly and syrphid flies, were the dominant floral visitors of *P. alpina.*



Appendix S2. Details of studied populations

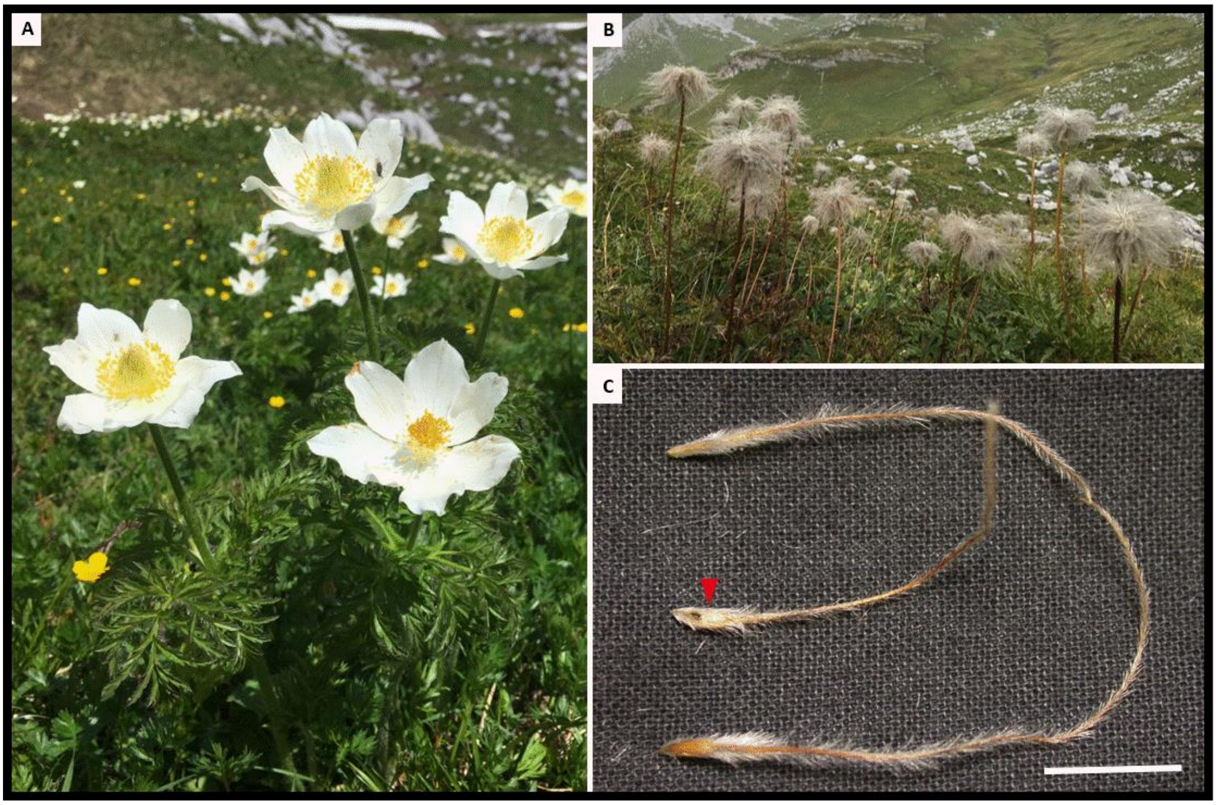
* Flowering season indicates whether the population starts flowering in late May to early June (E) or late June to July (L).

+ Grazing is quantified in terms of three categories, based on personal observation in 2019, i.e., no grazing (0), highly grazed (1), and intermediate level (0.5).

[!] Measurement was not conducted in Population LL1 due to a landslide in 2020 and in Population S1 due to unintended herbivory at the end of the growing season in 2021.

Population	Location	Site	GPS position	Altitude (a.s.l.)	Flower season *	Grazing [+]	Habitat	Note	Population survey[!] (2021)	Floral correlation (2021)	Leaf removal (2020-2021)	Selection gradient (2019)
S1	Solalex	Solalex1	46°17′36N 7°09′11″E	1723	E	0	open grassland	fenced		O	O	O
S1+	Solalex	Solalex1	46°17′42″N 7°09′09″E	1758	E	1	open grassland	shallow soil	O			
S2	Solalex	Solalex2	46°16′37″N 7°09′32″E	2122	L	0.5	open grassland	steep	O			O
S4	Solalex	Solalex4	46°16′42″N 7°09′47″E	2003	L	1	open grassland	flat, shallow soil	O			
B1	Bretaye	Bretaye	46°19′31″N 7°05′00″E	1818	E	0	open grassland		O			
LS1	Leysin	Leysin1	46°21′42″N 7°00′13″E	1995	L	0.5	open grassland		O			
LS2	Leysin	Leysin2	46°21′40″N 7°00′15″E	2006	E	0	open grassland	shallow soil	O			
R1	Rochers de Naye	Rochers de Naye	46°25′58″N 6°59′04″E	1953	E	0	open grassland	steep, fenced	O			
R2	Rochers de Naye	Rochers de Naye	46°25′58″N 6°59′03″E	1944	L	0.5	open grassland		O			
LM1	Les Mosses	Les Mosses	46°23′57″N 7°04′52″E	1694	E	1	enclosed grassland		O			
LL1	Lac Lioson	Lac Lioson	46°23′08″N 7°07′23″E	1951	E	0.5	open grassland	steep				O
LL3	Lac Lioson	Lac Lioson	46°23′05″N 7°07′25″E	1900	L	0.5	open grassland		O		O	
LL4	Lac Lioson	Lac Lioson	46°22′57″N 7°07′11″E	1983	L	0.5	open grassland		O			O

Appendix S3. Images of *Pulsatilla alpina* in the field and different categories of achenes. (A) A flowering individual. (B) Elongated stalks bearing achenes before dispersal at the end of the growing season. (C) Different categories of achenes. From the top to the bottom is an unfertilized achene, a predated achene, and a mature achene. Top achene: the color of unfertilized achenes is usually pale brown to yellow. The head part is flat and soft. Middle achene: Predated achenes have the same color and size as mature ones but have a hole made by seed predators (indicated by a red triangle). When squeezed, they are soft and empty. Bottom achene: Mature achenes are robust and contain a seed within a brown-dark head capsule. The heads of the achenes are swollen and hard. The white bar in (C) indicates one centimeter.



Appendix S4. Results of the linear mixed model assessing the effects of the intrinsic and extrinsic factors on stalk height at the end of the growing season across eleven populations.

Notes: ∗ $P < 0.05$, ∗∗ $P < 0.01$, ∗∗∗ $P < 0.001$

Fixed effect	**df**	**Likelihood-ratio test**	***P***
Total flower number	1	68.4	***
Vegetation height at early season	1	0.27	ns
Vegetation height at late season	1	9.66	**
Population	10	55.0	***
Random effect	**N**	**Variance component**	**SD**
Residuals		43.7	6.61
Transect	41	51.3	2.44

Appendix S5. Results of the linear mixed model assessing the effects of leaf removal treatment and population on stalk height.

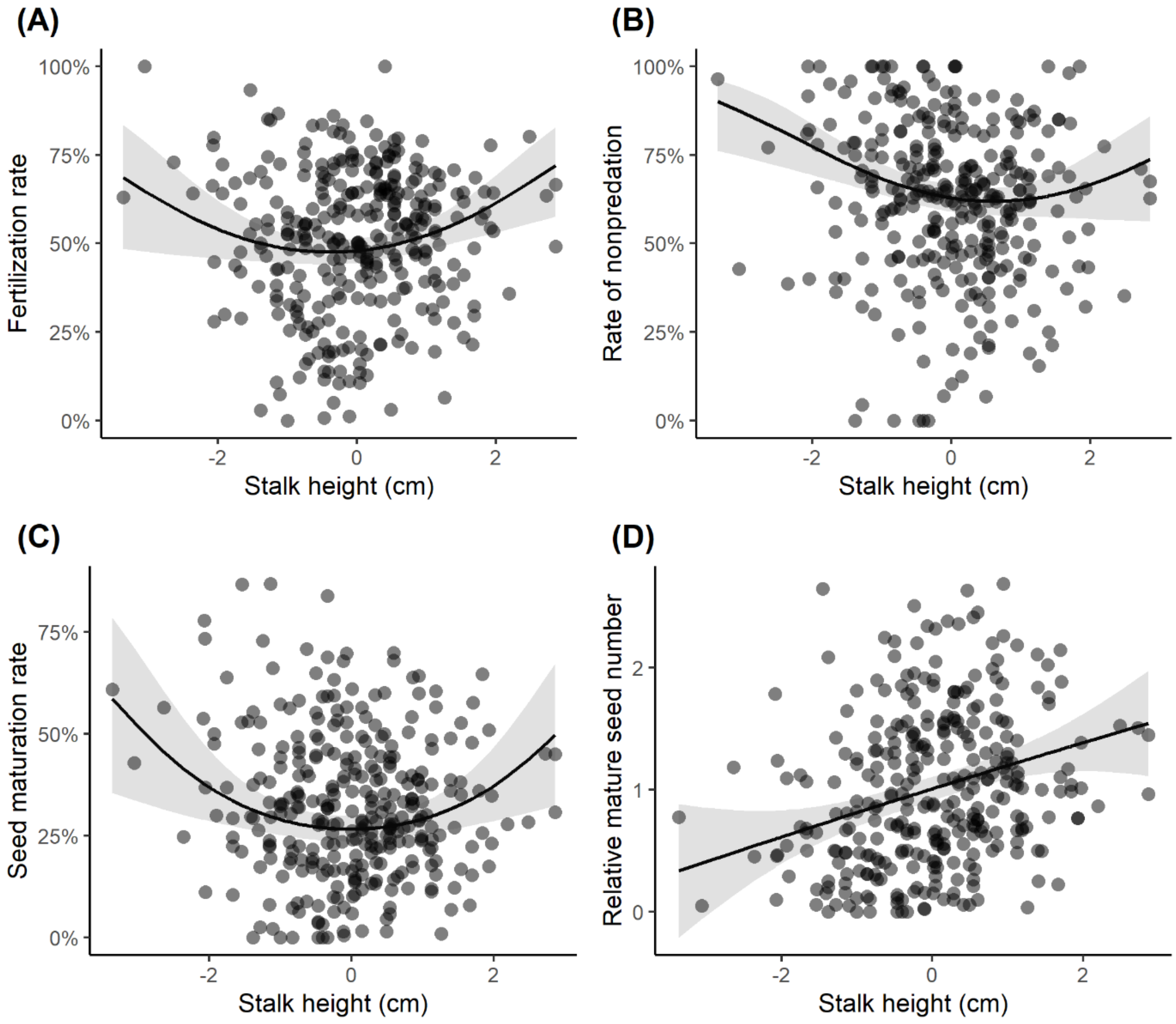
Notes: ∗ P < 0.05, ∗∗ P < 0.01, ∗∗∗ P < 0.001

Fixed effect	**df**	**Likelihood-ratio test**	***P***
Leaf removal	1	9.05	**
Population	1	5.02	*
Leaf removal: Population	1	0.83	ns
Random effect	**N**	**Variance component**	**SD**
Residuals		51.3	7.16
Individual	63	50.7	7.12

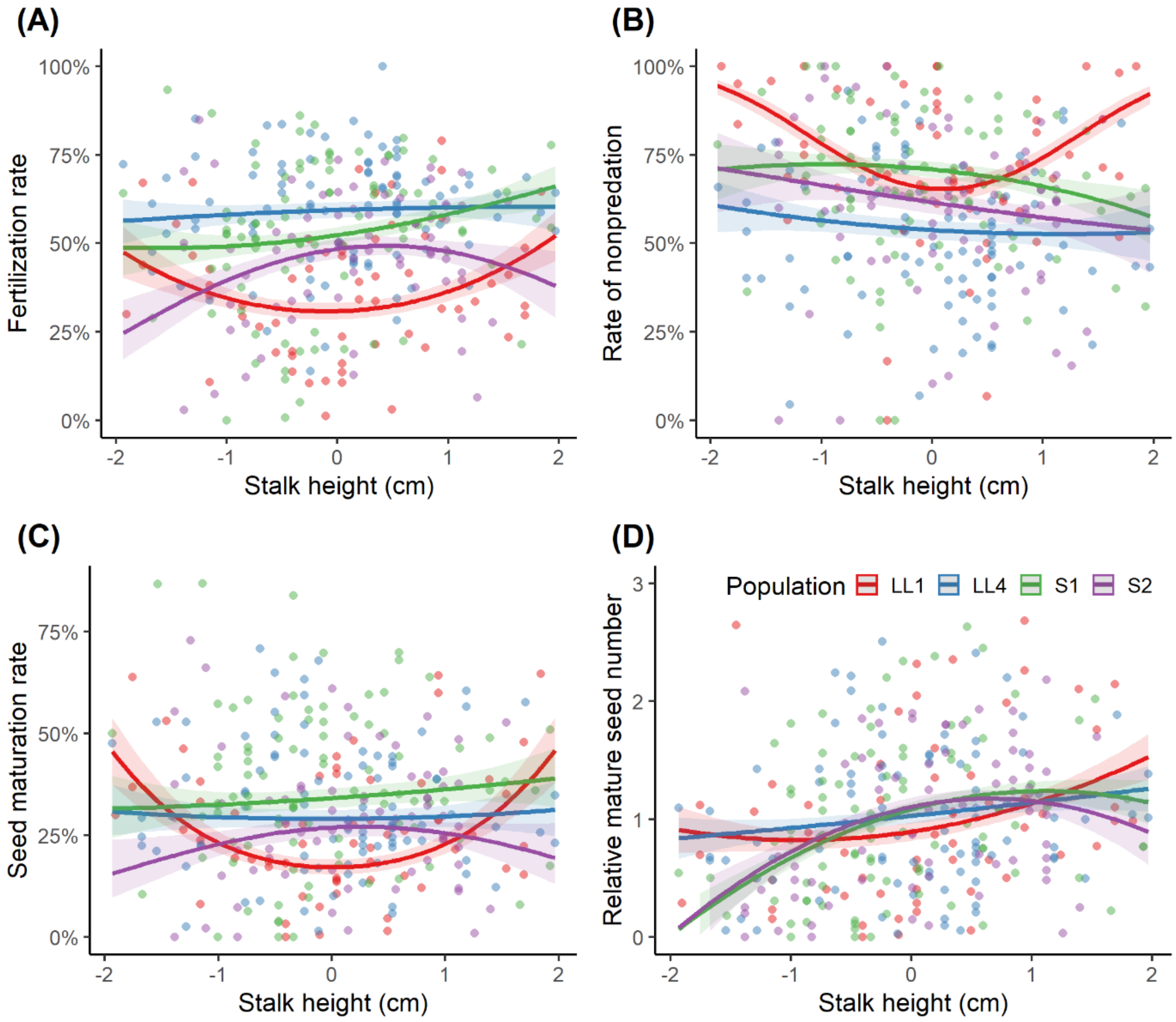
Appendix S6. Mean values and standard deviations for the four female fitness components and stalk height of the four populations. The mean and standard deviation were calculated at the flower-level in the four populations: S1 (*N* = 95 flowers), S2 (*N* = 74 flowers), LL1 (*N* = 62 flowers), and LL4 (*N* = 91 flowers).

Population	Fertilization rate	Rate of non-predation	Seed maturation rate	Mature seed number	Stalk height
LL1	0.37 ± 0.18	0.71 ± 0.22	0.27 ± 0.16	51.2 ± 35.9	43.7 ± 6.68
LL4	0.60 ± 0.16	0.53 ± 0.20	0.33 ± 0.17	72.2 ± 42.9	45.9 ± 7.69
S1	0.53 ± 0.21	0.66 ± 0.21	0.38 ± 0.20	71.8 ± 45.9	43.5 ± 7.51
S2	0.49 ± 0.19	0.59 ± 0.22	0.30 ± 0.17	60.0 ± 36.9	36.9 ± 7.18

Appendix S7. Plots of the single (generalized) linear mixed models assessing the general effects of standardized stalk height on female fitness components. The relationship between stalk height at the end of the flowering stage and (A) fertilization rate, (B) rate of nonpredation, (C) seed maturation rate, and (D) relative mature achene number from single regression models. Gray points are flowers from the four populations studied in 2019. Interactions between stalk height and population are all nonsignificant. The shaded ribbon indicates the 95% confidence interval of the regression lines.



Appendix S8. Visualization of the relationship between standardized stalk height at the end of the flowering stage and (A) fertilization rate, (B) nonpredation rate, (C) seed maturation rate, and (D) relative mature seed number among populations from single regression models evaluating the selection differentials without extreme phenotypes (i.e., phenotypes larger or small than two standard deviations from the mean value of each population; 14 data points removed from a total of 322 data points). Raw data points and regression lines of the studied populations are shown in different colors. The shaded ribbons indicate the standard error of the regression lines. The results indicate that the selection differentials and gradients estimated might be largely driven by those extreme phenotypes. However, it seems the extreme phenotypes have shaped the selection in the same direction in all four populations (see Fig. 4 for a comparison). The linear and quadratic differentials for fertilization rate, nonpredation rate, and seed maturation rate were nonsignificant in all the populations except the quadratic coefficients in LL1 population ($P < 0.001$, $P = 0.05$, and $P < 0.01$ in the three fitness components, respectively). For relative mature seed number, the quadratic coefficients were all nonsignificant, while the linear coefficients were all significant except for LL1 (marginally n.s., $P = 0.06$) and LL4 (n.s., $P = 0.18$) population. Stronger approaches one can use in further studies to overcome the issue are either to manipulate and create extreme phenotypes or to select extreme phenotypes from the natural variation to have a better estimate of the selection at the two ends of the phenotypic range.


(A)
Fertilization rate
100%
75%
50%
25%
0%
-2
-1
0
1
2
Stalk height (cm)
(B)
Rate of nonpredation
100%
75%
50%
25%
0%
-2
-1
0
1
2
Stalk height (cm)
(C)
Seed maturation rate
75%
50%
25%
0%
-2
-1
0
1
2
Stalk height (cm)
(D)
Relative mature seed number
3
2
1
0
Population
LL1
LL4
S1
S2
-2
-1
0
1
2
Stalk height (cm)

Appendix S9. Results of the generalized linear mixed models assessing the effects of four floral traits and population on female fitness components.

Notes: ∗ $P < 0.05$, ∗∗ $P < 0.01$, ∗∗∗ $P < 0.001$

	Response variable											
	Fertilization rate (binomial)			**Rate of nonpredation (binomial)**			**Seed maturation rate (binomial)**			**Relative mature seed number (normal)**		
Fixed effect	**df**	**LRT**	***P***	**df**	**LRT**	***P***	**df**	**LRT**	***P***	**df**	**LTR**	***P***
Stalk height	1	0.44	ns	1	0.00	ns	1	0.19	ns	1	0.26	ns
Tepal length	1	5.58	*	1	1.48	ns	1	7.26	**	1	4.32	*
Pistil number	1	0.00	ns	1	30.0	***	1	6.84	**	1	85.6	***
Stamen number	1	9.88	**	1	3.69	.	1	12.5	***	1	5.04	*
Stalk height^2	1	5.86	*	1	3.90	*	1	7.79	**	1	2.57	ns
Tepal length^2	1	0.57	ns	1	0.07	ns	1	0.37	ns	1	0.46	ns
Pistil number^2	1	0.43	ns	1	1.86	ns	1	1.55	ns	1	9.43	**
Stamen number^2	1	4.53	*	1	0.05	ns	1	2.25	ns	1	2.26	ns
Population	3	35.1	***	3	21.5	***	3	9.40	*	3	0.24	ns
Stalk height: Population	3	0.54	ns	3	6.73	.	3	1.13	ns	3	2.04	ns
Tepal length: Population	3	1.47	ns	3	4.63	ns	3	2.18	ns	3	1.61	ns
Pistil number: Population	3	1.58	ns	3	13.6	**	3	3.08	ns	3	1.37	ns
Stamen number: Population	3	1.83	ns	3	4.90	ns	3	4.81	ns	3	5.30	ns
Stalk height^2: Population	3	3.14	ns	3	6.71	.	3	2.44	ns	3	3.24	ns
Tepal length^2: Population	3	3.17	ns	3	3.70	ns	3	4.28	ns	3	3.59	ns
Pistil number^2: Population	3	0.92	ns	3	0.84	ns	3	1.90	ns	3	9.86	**
Stamen number^2: Population	3	4.59	ns	3	3.80	ns	3	6.54	.	3	3.73	ns
Random effect	**N**	**Variance component**	**SD**	**N**	**Variance component**	**SD**	**N**	**Variance component**	**SD**	**N**	**Variance component**	**SD**
Flower ID	322	0.52	0.72	321	0.65	0.81	322	0.63	0.79			
Individual ID	151	0.14	0.37	151	0.21	0.46	151	0.22	0.47	151	0.09	0.29

GENERAL DISCUSSION

In this thesis, using as a model the alpine perennial herb *Pulsatilla alpina*, a species with enormous variation in its sex allocation, I investigated (1) how sex allocation and reproductive success depend on life history and temporal changes in sexual selection, (2) how reproductive success depends on floral sex allocation, taking into account the effect of mating system, and (3) how an ancillary trait, i.e., the height of floral stalks, is under the selection of different female fitness components by pollinators and seed predators. In this final chapter, I discuss the main conclusions drawn from my results as a whole and consider some of the unanswered questions that deserve further investigation.

Main conclusions

In **Chapter I**, by considering the effects of plant resource status and mating opportunity jointly, I revealed and was able to interpret the size- and time-dependent sex allocation strategy *in P. alpina* as a case of gender-diphasy and andromonoecy. Neither of these strategies are directly predicted by the theories that consider the two factors independently (Brunet and Charlesworth, 1995; Klinkhamer et al., 1997; Cadet et al., 2004). The estimate of male reproductive success using a mass-action model based on detailed phenology records further provides adaptive explanations for the sex allocation strategies, in which small individuals likely maximize their lifetime fitness by producing early male flowers to capitalize on the high mating opportunity in the early season due to protogyny in the species. Moreover, the results point to a plausible hypothesis that andromonoecy may represent a resolution of the sexual conflict due to dichogamy (Schlessman, 2010). This work highlights the utility of considering both life history and sexual selection to study sex allocation strategies.

In **Chapter II**, I conducted a detailed examination of reproductive success in a population of *P. alpina* by applying novel approaches to analyzing the dependency of reproductive success on sex allocation. My study here provided unusual insights into important aspects of a general understanding of sex allocation. Taking advantage of a predominantly single-flowered population and with the aid of genetic markers, I was able to assign reproductive success explicitly, especially through male function, to different sex-allocation phenotypes, and to take into account the effects of the mating system. I showed that female reproductive success depends on male allocation likely as a consequence of seed discounting, a factor that is not considered in models that explain reproductive strategies as a function of a simple allocation trade-off between sexual functions (Charlesworth and Charlesworth, 1981; Charnov, 1982). Moreover, mapping reproductive success onto a fitness landscape as a function of both female and male allocation as partly independent variables showcases a new way to study sex allocation in hermaphroditic organisms. This approach is particularly promising for future work because it bypasses the dilemma of choosing currencies of allocation and moves beyond the univariate point of view of the 'fitness gain curve' (Charlesworth and Morgan, 1991; Campbell, 2000). Most importantly, the approach demonstrated a rugged fitness landscape of total reproductive success that helps to explain the andromonoecious sex allocation strategy in *P. alpina*.

In **Chapter III**, I considered the implications of allocation to a costly secondary sexual trait, floral stalk height, which is thought to facilitate seed dispersal by wind in *P. alpina*. Here, I used various approaches to study factors governing the expression and selection of the trait. The results revealed that (1) stalk height was tightly associated with female allocation of flowers, (2) that the expression of the trait was governed by both intrinsic resource status and extrinsic vegetation height, (3) that stalk height is likely a substantially costly trait, and (4) that stalk height is under disruptive selection by pollinators and seed predators in terms of female fitness components. These results imply that the height of floral stalks should be taken into account when considering the andromonoecious sex allocation strategy of the species, given its association with female allocation and reproductive success and its implications for resource costs. This work points to the importance of considering not only reproductive traits but also ancillary traits when studying sex allocation.

It is worth noting that the research presented in this thesis contributes to the very few empirical studies that have convincingly examined sex allocation theories in wild populations of hermaphroditic plants using estimates of both male and female reproductive success (Campbell, 2000). To address one of the greatest challenges for studies of sex allocation, i.e., to quantify male reproductive success (Emms, 1993; Campbell, 2000; Thomson, 2006), I adopted two different methods: a mass-action model (**Chapter I**); and a genetic paternity analysis (**Chapter II**). The former approach provided useful insights into how reproductive success depends on plant size and time based on a few assumptions, whereas the latter allowed me to examine the dependency of reproductive success on sex allocation in substantial detail. Interestingly, although the two populations used in the two studies differed in many aspects, e.g., population size and density, the two analyses yielded considerably similar results (Appendix S1), reinforcing the conclusions drawn from each of the two studies. Importantly, the complexity of the dependency of female reproductive success on sex allocation, in particular, has long been overlooked (Campbell, 2000). The studies in this thesis considering the effects of the mating system (**Chapter II**) and biotic interactions (**Chapter III** and the discussion in the next section) call for attention to this complexity when quantifying female reproductive success.

As a summary of the findings from my studies in *P. alpina*, it is tempting to postulate a hypothesis for the evolution of andromonoecy and gender-diphasy that may be generally applicable to other perennial dichogamous herbaceous plants. First of all, strong dichogamy, i.e., a separation of sexual functions in time within hermaphroditic flowers, expressed in species with a relatively short flowering season, inevitably leads to a variation in mating opportunity over the course of a flowering season (Brunet and Charlesworth, 1995) and thus creates a potential sexual conflict between the two sexes within flowers. For instance, in protogynous species, in which the female function precedes the male function, it is advantageous to start the male function early in the season as the mating opportunity decreases across the season. Thus, gradually reducing the allocation to the female function in a flower likely enhances reproductive success via the male function (Brunet and Charlesworth, 1995). Nonetheless, if a reduction in female allocation, or an increase in male allocation, leads to elevated seed discounting within a flower, and if it is accompanied by a higher resource cost in female function per unit, plants may be selected to produce qualitatively male-only flowers, i.e., an andromonoecious sex allocation strategy (Spalik, 1991; de Jong et al., 2008). Furthermore, in perennial herbs, which draw resources for reproduction and growth from a storage tissue every season, producing only one or a few male flowers when the resource status of the individual is low may maximize lifetime fitness as a result of trade-offs among reproduction, growth, and survival (Zhang and Jiang, 2002). As a consequence, individuals may shift their gender between a male-phase and a hermaphrodite-phase, i.e., they may express ‘gender-diphasy’ (Schlessman, 1988). This hypothesis may explain the prevalence of andromonoecy and gender-diphasy in species from Apiales (Schlessman, 2010), Lilliales (Peruzzi, 2012; Zhang et al., 2014), and Ranunculales (Pellmyr, 1987; Lindh, 2017) with characteristics similar to those of *P. alpina*, i.e., expressing strong dichogamy, a relatively short flowering season, a greater resource cost of female allocation, and a perennial herbaceous life history.

Unanswered questions, and some preliminary results

Considering to complete the understanding of the evolution and ecology of plant sex allocation strategies, there are a few questions beyond the time constraints of this thesis that deserve further investigation. Below, I briefly discuss the key puzzling questions and bring up some results of preliminary investigations I have conducted but which have not found space in the thesis itself.

Cost of female and male functions

In perennial polycarpic plants, the optimal sex allocation strategy depends on not only the reproductive success gained via the two sex functions in the current season, but also the fitness implications derived from between-season trade-offs among reproduction, growth, and survival (Figure 1; Zhang and Jiang, 2002). Indeed, what natural selection is optimizing is the ‘lifetime’ fitness output of organisms under different ecological and evolutionary settings. In other words, to minimize the marginal cost in terms of fitness when allocating resources to different functions. Thus, studies of sex allocation should be put into a broader context – beyond solely focusing on female and male sex allocation and reproductive success in a single year.

I believe that *P. alpina* could potentially serve as a useful model system to continue to address the key questions of sex allocation strategies of perennial plants (Figure 1). First of all, the plant regenerates its aboveground parts every growing season from an overwinter storage tissue, which allows us to feasibly quantify the resource status, i.e., the storage tissue, before or after the allocation decisions made each season. Second, the allocation to different functions, e.g., reproduction via female and male functions and growth, can be feasibly quantified, as has been done in this thesis. Third, experiments of quantifying actual resource status and monitoring sex allocation decisions of the same individuals over several years could be useful to discern the trade-offs in the sex allocation strategy of perennials. My explanation of the gender-diphasic sex allocation strategy in *P. alpina* is largely based on a greater marginal cost of the female than the male function for transmitting genes to the next generation, especially when the resource status of an individual is small. It will be interesting first to measure directly the explicit cost, e.g., non-structural carbohydrates, of male and female functions via manipulations such as bud removal. Second, by following a number of individuals over several years, the implicit cost of female versus male functions in terms of growth and survival could be clarified.

Taking advantage of the remarkable variation and experimental accessibility of *P. alpina* as a model, I have been following approximately a hundred individuals in one population over the course of four seasons, and I will now measure the non-structural carbohydrates of manipulated individuals this season. I expect results from this investigation will yield further insights into the relationship between the marginal costs of reproduction and the realized sexual system and life history expressed in natural populations.

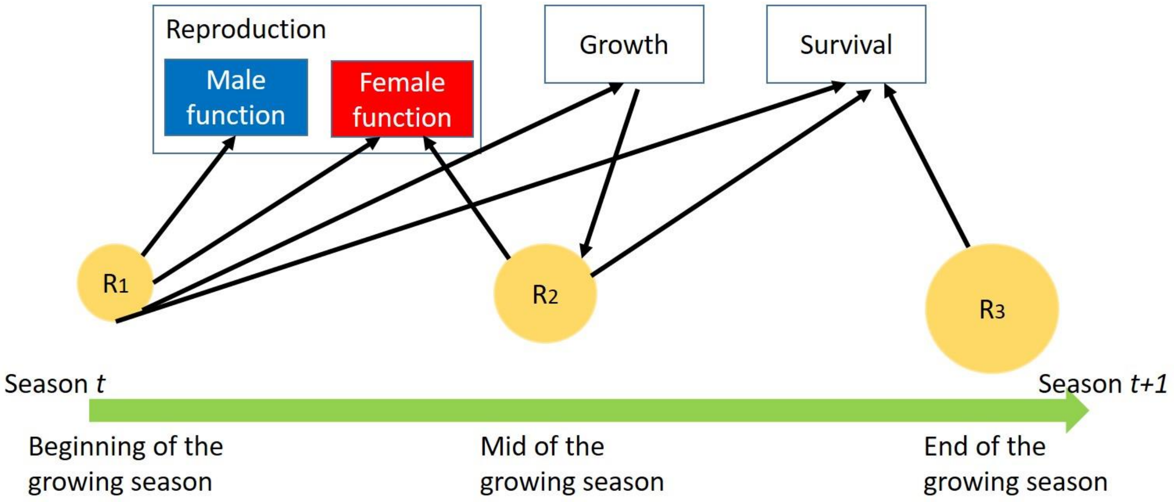


Figure 1. A conceptual diagram showing the dynamics of resource allocation to different functions across a growing season *t* in perennial polycarpic herbaceous plants, e.g., *Pulsatilla alpina*. R_1, R_2, and R_3 represent different resource statuses of an individual through a growing season. Reproduction, growth, and survival represent three non-exhaustive examples of biological functions to which a plant needs to allocate its resource, and thus there are potential trad-offs. The black arrows indicate the hypothetic direction of resource flows.

Biotic interactions with plant sex allocation

The evolution of sex allocation strategies should depend on, and respond to, a wide number of factors affecting the life history, resource acquisition, and fitness gains of individuals. Some of these have been considered in this thesis, but I have not considered in any detail one important factor: the role of biotic interactions. The shape of fitness gain curves is generally thought to be shaped by mutualistic insect pollen dispersers and animal seed dispersers (Charlesworth and Morgan, 1991; Campbell, 2000). Some of my results in this thesis are directly dependent on such interactions. However, *P. alpina* also interacts with other aboveground herbivores, pre-dispersal seed predators and their predators, and belowground symbioses that likely also affect the ecology and evolution of sex allocation.

Aboveground herbivores can directly damage the vegetative tissues, reduce the resource status of the individuals, and thus affect not only the sex allocation decisions of individuals in the following seasons but also the operational sex ratio and the intensity of sexual selection in the population. In plants with size-dependent sex allocation like *P. alpina*, herbivory is likely to cause a male-biased operational sex ratio as a short-term ecological effect, as was found in a study of *Arisaema* by Bialic-Murphy et al., (2020). In a study in parallel to my thesis work, I found that simulated herbivory on leaf tissues led to male-biased sex allocation after only one season (Appendix S2D) and that herbivory intensity by cattle and the operational sex ratio in *P. alpina* vary over populations and years (Appendix S3). Moreover, I have set up a long-term herbivore-exclusion experiment to measure the accumulated effects of ongoing herbivory in natural populations. I hypothesize that the male-biased operational sex ratio in some populations is mostly a maladaptive consequence of the size-dependent sex allocation strategy of the species under recent human activity (pasturing) in the Anthropocene. It remains to be seen whether the populations show evolutionary responses in life history or sex allocation traits to altered operational sex ratios due to herbivory.

Discussion of ecological factors shaping the fitness gain curves has rarely gone beyond mutualistic biotic interactions, e.g., with pollinators or seed dispersers. Indeed, antagonists and their predators may affect a plant's reproductive success and determine the shape of the gain curves as well (Ashman, 2002; Strauss and Irwin, 2004; Strauss and Whittall, 2006; Johnson et al., 2015). My observations suggest that seed predation by *Phytomyza* larvae is greater in flowers with more pistils, likely leading to a saturating female gain curve in years when the seed predators are abundant (Appendix S4). These observations have implications for how hermaphroditic individuals should allocate their resources to different flowers, i.e., a sex allocation decision at the flower level. Interestingly, interactions with pollinators did not show any dependency on female sex allocation and thus did not alter the shape of the female gain curve. Moreover, the intensity of seed predators saturating the female gain curve appears to depend greatly on the abundance of the seed predators in each season and population (see **Chapter II**). Thus, how spatial and temporal variation in the antagonistic interaction with seed predators shapes the sex allocation strategy remains to be investigated. Lastly, during my fieldwork, I found a highly specialized tri-trophic interaction among *P. alpina*, the seed predator, and a parasitoid that parasites the larvae of the seed predators during the fruiting stage of *P. alpina*. How this extra layer of complexity affects the reproductive success and the fitness gain curves in *P. alpina* also remains to be explored in detail.

Finally, in a collaborative project with a postdoc and master student in Prof Sanders' lab, we found that *P. alpina* individuals in different gender phases were associated with different colonization rates of arbuscular mycorrhizal fungi (AMF), and we are now investigating the AMF communities of different gender phases (Appendix S5). Plants interact with various belowground microbes, among which arbuscular mycorrhizal fungi (AMF) are mutualistic symbionts that assist nutritional uptake of the plants from the soil in exchange for carbon resources (Parniske, 2008). As a result, AMF may play an important role in the acquisition of micronutrients and in the allocation of the photosynthetic products of its host (Johnson, 2010). As reproduction through the female and male function usually rely on different sets of micronutrients in plants (Obeso, 2002), it has been found that males and females in a few dioecious species are associated with different AMF communities, likely as a result of sexual specialization in terms of physiology (Varga, 2010). However, the association has never been investigated in species showing gender-diphasy, i.e., the gender types are not genetically fixed. Although we are currently unable to draw any conclusion based on our preliminary analysis of the colonization rate, it will be of substantial further interest to investigate this intriguing intersection between the fields of plant sex allocation and plant-microbiome interactions.

Concluding remarks

In a conclusion, the results of my research demonstrate an unusual empirical examination of sex allocation theories incorporating life history, sexual selection, and mating systems together in *P. alpina*. Moreover, they provide not only adaptive explanations to the evolution of sex allocation strategies of gender-diphasy and andromonoecy but also valuable insights into the general understanding of sex allocation in hermaphroditic plants. To have a more delicate understanding of different sex allocation strategies in hermaphroditic plants, sex allocation should not be viewed as simply a matter of optimizing reproductive success in a trade-off of allocating resources to the two sex functions. Furthermore, more attention should be focused on mechanisms that determine the complex dependency of reproductive success on sex allocation.

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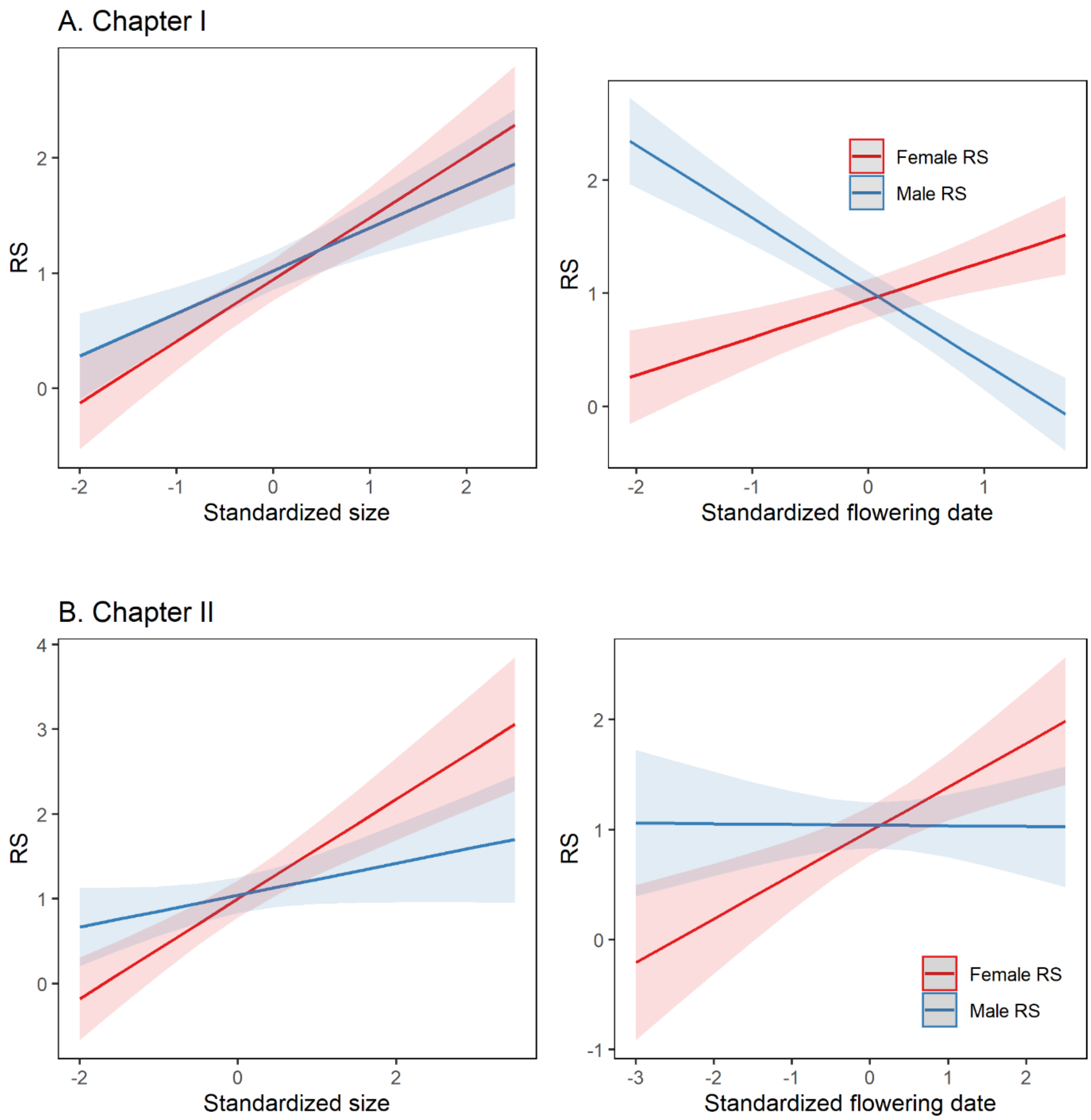
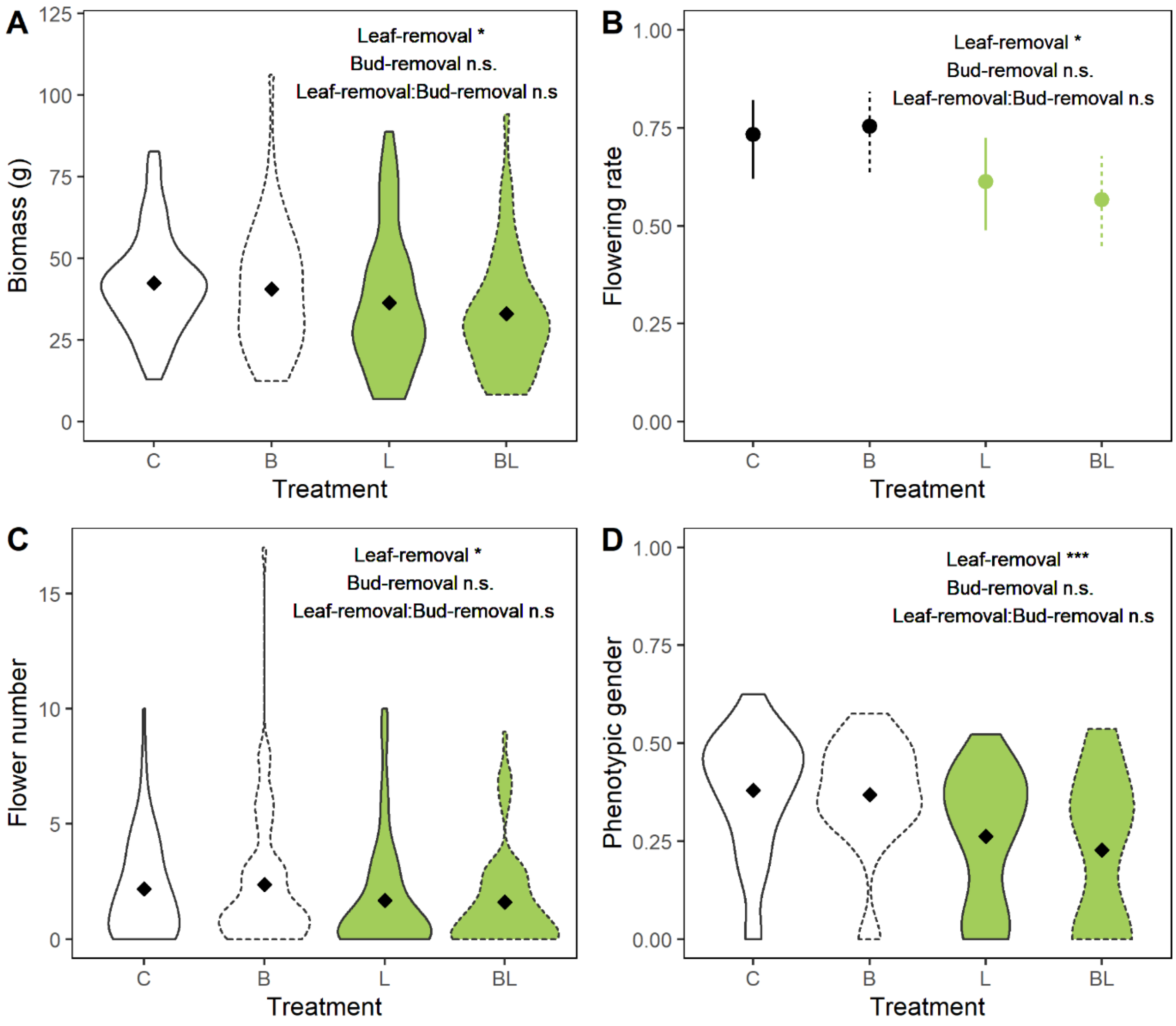
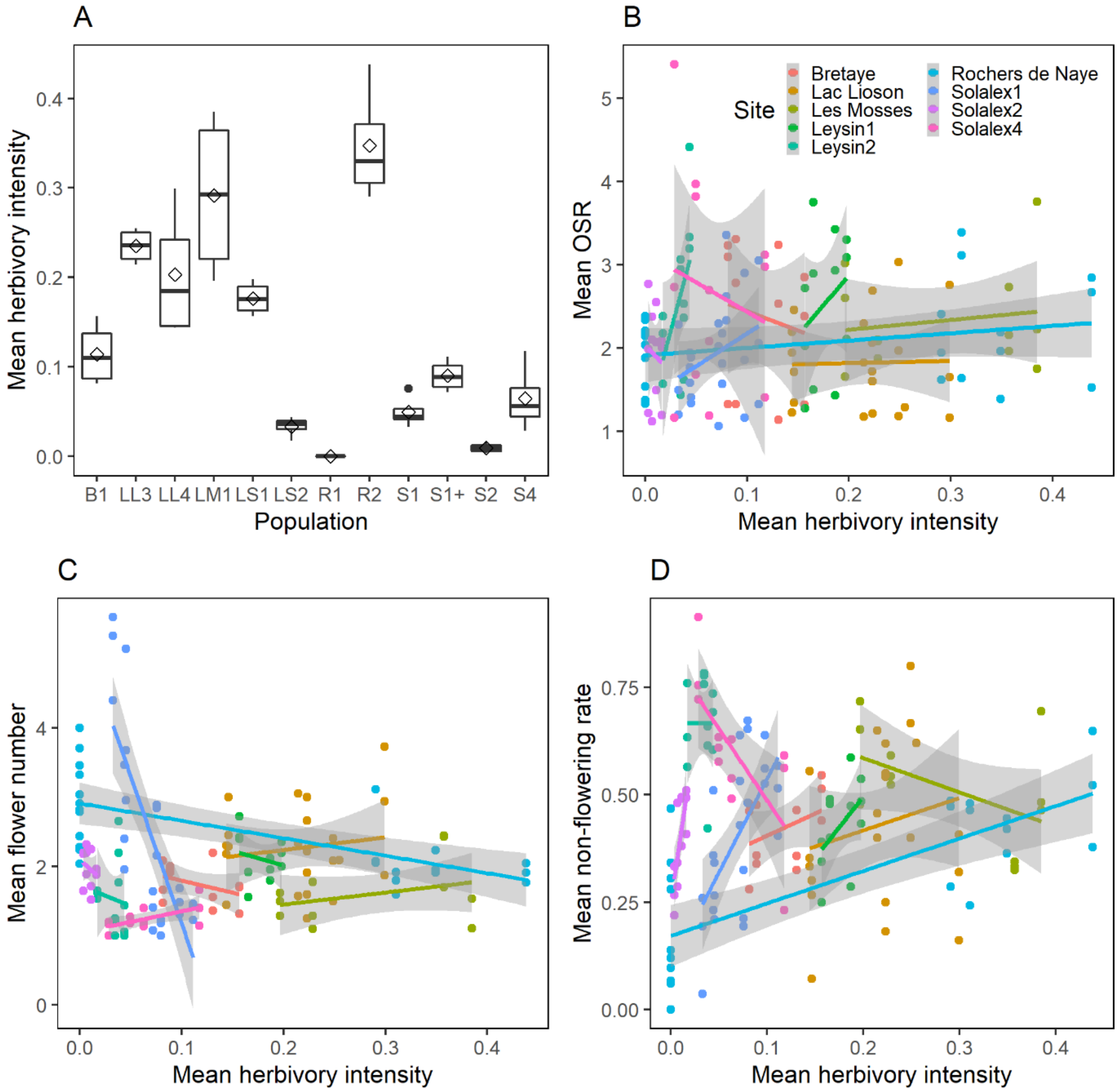
APPENDIX



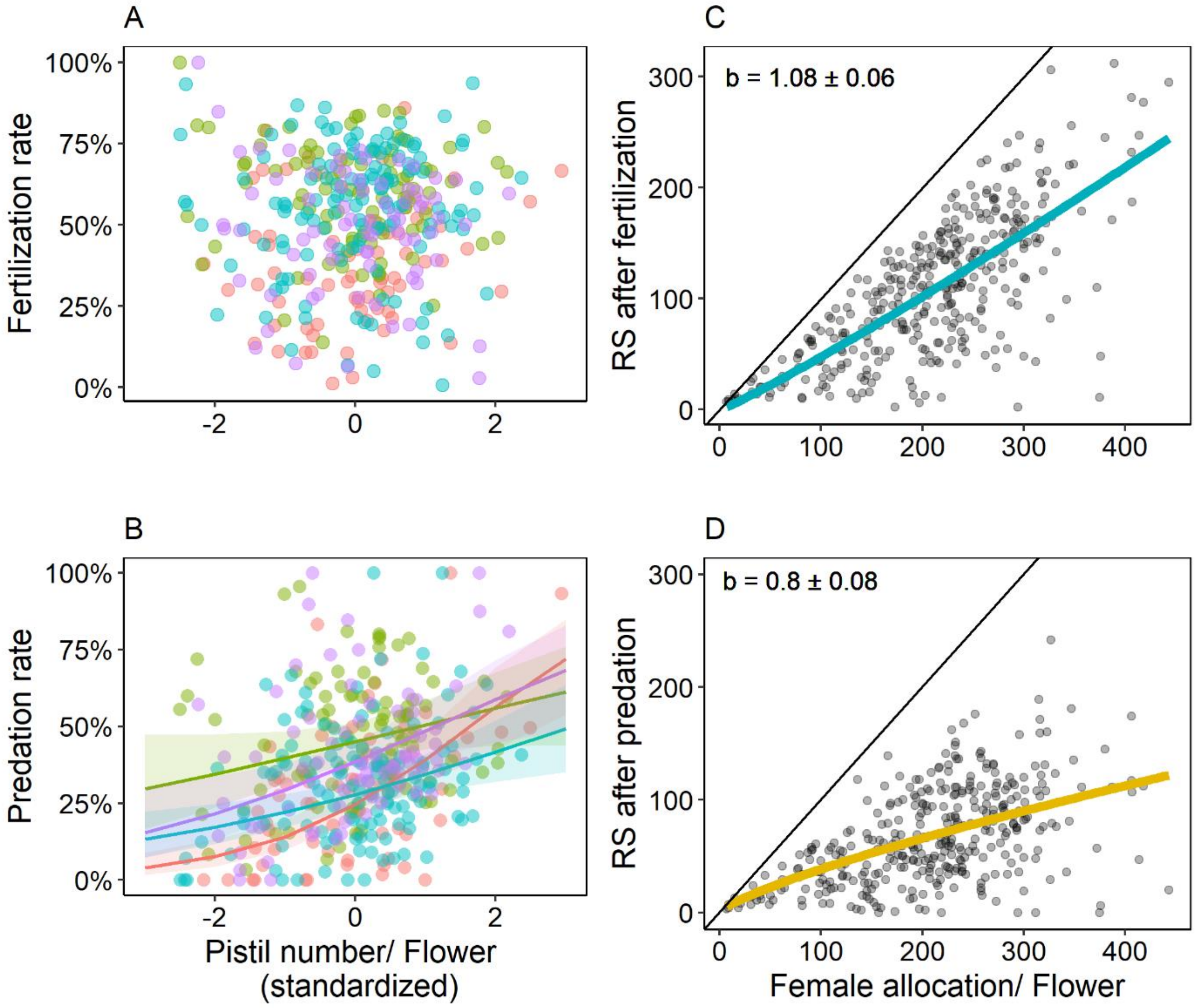
Appendix S1. Comparison of the dependency of reproductive success at the individual level on size and timing between two studies using prospective siring success (A, see **Chapter I**) and paternity analysis (B, see **Chapter II**), respectively, to estimate male reproductive success. The data from the two studies were analyzed by two generalized least square models (*gls*) with the same formula used in **Chapter I**. (B) plants size and flowering date of the individuals were calculated with the same methods used in **Chapter I**. The sample size was 88 and 125 individuals in (A) and (B), respectively.



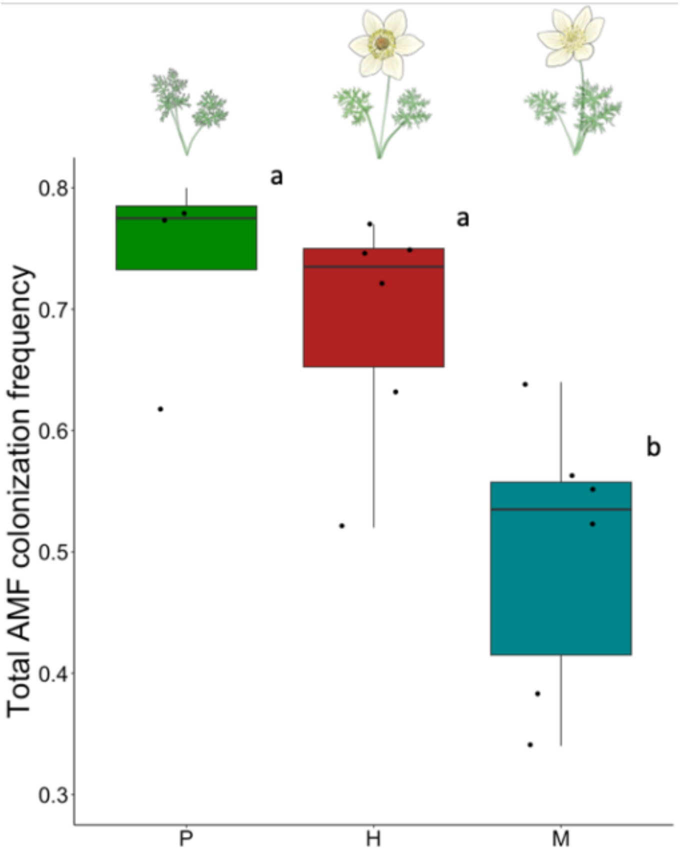
Appendix S2. Effects of simulated herbivory of leaves and buds on aboveground biomass (A), the flowering rate (B), flower number (C), and phenotypic gender (D). Four treatments are the control (C, *N* = 71), bud-removal (B, *N* = 65), leaf-removal (L, *N* = 62), and bud- and leaf-removal (BL, *N* = 67). Treatments with leaf-removal manipulation are colored green. Treatments with bud-removal manipulation are in the dotted line. In (B), the flowering rate was the proportion of flowering individuals in the next season after treatments. Means and 95% confidence intervals of each treatment are shown. In (D), phenotypic gender was calculated in terms of femaleness. In (A), (C), and (D), the black dot denotes the mean of each treatment and the distribution of data was shown in violin plots.



Appendix S3. (A) Mean herbivory intensity of 12 populations from 2020 to 2022. In each population, around three to four permanent transects were set (see Materials and Methods of **Chapter III**). Herbivory intensity was quantified at the end of the growing season by assigning all the individuals within the transect into four categories of herbivory intensity representing 0, 0 to 33, 33 to 66, and 66 to 100 percent of damage in aboveground tissues by herbivores. Plots showing the preliminary exploration of the relationship between mean herbivory intensity and mean operational sex ratio (OSR) (B), mean flower number (C), and mean non-flowering rate (D) over three years at the transect level in different sites. In (B), (C), and (D), transects from populations located at the same slope are grouped into the same sites (see Appendix S2 of **Chapter III**). OSR was quantified at the beginning of the flowering season by assigning all the flowers in the transects into four categories of sex allocation (see Materials and Methods of **Chapter I**) and then dividing the total stamen number by the total pistil number produced in the transects. Although a positive correlation in OSR (A), non-flowering rate (C), and a negative correlation in flower number (B) with herbivory intensity were expected based on the simulated herbivory experiment at the individual level, the patterns shown by the preliminary analysis indicate a much more complicated scenario likely involving interactions among the timing of the herbivory, local abiotic factors, and evolutionary responses. I am currently seeking more adequate methods to analyze the data.



Appendix S4. Plots showing the dependency of fertilization rate (A) and seed predation rate (B) on pistil number of flowers resulting in linear (C) and saturating (D) female gain curves, respectively. Data presented in four different colors in (A) and (B) was derived from the four populations used in the phenotypic selection analysis in **Chapter III**. (A) the fertilization rate did not depend on the pistil number. (B) the seed predation rate positively depended on pistil number in all the populations. Exponents of the female fitness gain curves after fertilization (C) and seed predation (D) were derived from a power function and depicted by a blue and yellow line, respectively. The exponent in (D) was significantly less than one.



Appendix S5. Total AMF colonization frequency of root samples from *P. alpina* individuals at different phases. Non-flowering phase, hermaphrodite-phase, and male-phase individuals are indicated by P (*N* = 3), H (*N* = 6), and M (*N* = 6), respectively. Colonization rates of phases having the same letter are not significantly different. The colonization rate was calculated by the presence or absence of AMF structure (hyphae/vesicles/arbuscules) over 100 observations of each individual. The project is a collaboration with Prof Ian Sanders, Dr Soon-Jae Lee, and Fabio Opreni. The figure is modified with permission from Fabio Opreni.