Disentangling the effects of jasmonate and tissue loss on the sex allocation of an annual plant

Nora Villamil*, Benoit Sommervogel and John R. Pannell

Department of Ecology and Evolution, Université de Lausanne, Lausanne, Switzerland

Selection through pollinators plays a major role in the evolution of reproductive traits. However, herbivory can also induce changes in plant sexual expression and sexual systems, potentially influencing conditions governing transitions between sexual systems. Previous work has shown that herbivory has a strong effect on sex allocation in the wind-pollinated annual plant *Mercurialis annua*, likely via responses to resource loss. It is also known that many plants respond to herbivory by inducing signaling, and endogenous responses to it, via the plant hormone jasmonate. Here, we attempt to uncouple the effects of herbivory on sex allocation in *M. annua* through resource limitation (tissue loss) versus plant responses to jasmonate hormone signaling. We used a two-factorial experiment with four treatment combinations: control, herbivory (25% chronic tissue loss), jasmonate, and combined herbivory and jasmonate. We estimated the effects of tissue loss and defense-inducing hormones on reproductive allocation, male reproductive effort, and sex allocation. Tissue loss caused plants to reduce their male reproductive effort, resulting in changes in total sex allocation. However, application of jasmonate after herbivory reversed its effect on male investment. Our results show that herbivory has consequences on plant sex expression and sex allocation, and that defense-related hormones such as jasmonate can buffer the impacts. We discuss the physiological mechanisms that might underpin the effects of herbivory on sex allocation, and their potential implications for the evolution of plant sexual systems.

KEYWORDS
herbivory, defoliation, jasmonate, anti-herbivore defenses, hormone, sex allocation, sexual system, *Mercurialis annua*

Introduction

Flowering plants are remarkable for the diversity of their reproductive structures (flowers and inflorescences) and their sexual systems, which range from simultaneous hermaphroditism to completely separate sexes (dioecy), including intermediate systems in which males or females co-exist with hermaphrodites (Barrett, 2002). Much of this
Herbivory has at least two consequences for plants. Most directly, it causes tissue loss, reducing plant size and limiting resources available for maintenance, growth and reproduction through male and female sexual functions (Karban and Strauss, 1993; Lehtilä and Strauss, 1997, 1999; Mothershead and Marquis, 2000; Hambäck, 2001; Poveda et al., 2003; Ivey and Carr, 2005; Núñez-Farfán et al., 2007; Kessler et al., 2011). If herbivory influences fitness through male versus female allocation differently, then selection should favor a norm of reaction that we might label “conditional sex allocation” (Freeman et al., 1980; López and Domínguez, 2003; Hirata et al., 2012), jasmonate is essential for male flower development, in tomato it is required for female flower development (Li et al., 2004), and in rice it determines the sex of the developing sexual organs (Acosta et al., 2009; Cai et al., 2014; Yuan and Zhang, 2015). Importantly, however, the effects of jasmonate on the female or male components of sexual development vary among species (Wasternack et al., 2013). For instance, in Arabidopsis thaliana (Browse, 2009) and in maize (Yan et al., 2012), jasmonate is essential for male flower development, in tomato it is required for female flower development (Li et al., 2004), and in rice it determines the sex of the developing sexual organs (Acosta et al., 2009; Cai et al., 2014; Yuan and Zhang, 2015).

In this study, we explored norms of reaction in sex expression and sex allocation to herbivory in an experiment designed to uncouple its direct (through tissue loss) and indirect effects (due to defensive jasmonate signaling). Classic plant resource allocation theory suggests that increased investment in reproduction and growth should come at a cost in allocation to defense, and vice versa (Herms and Mattson, 1992). We thus tested the hypothesis that herbivory should cause plants to reduce their reproductive effort through a trade-off between resources allocated to defense versus reproduction. We reasoned that defense-related traits, such as those induced by jasmonate signaling, may link the coordinated evolution of reproductive and
defensive traits. Growing evidence suggests that reproductive and defensive traits are not independent (Carr and Eubanks, 2014; Campbell, 2015; Johnson et al., 2015; Lucas-Barbosa, 2016), yet the effects of plant defensive strategies on trade-offs between male and female plant fitness and sex allocation still remain largely unexplored (García and Eubanks, 2019).

To uncouple the direct and indirect effects of herbivory on sex expression, and to test the role of jasmonate on conditional sex allocation, we conducted a two-factorial experiment manipulating tissue loss (25% chronic defoliation) and plant anti-herbivore defenses via the jasmonate pathway (external application of jasmonate), and measured the sexual expression of plants with both a male and a female function. In many species (Reymond and Farmer, 1998; Han, 2016; Wang et al., 2019), plants that are damaged automatically release jasmonate into the environment, and it is therefore impossible for them to experience tissue loss without jasmonate release. However, our experiment included all other biologically possible combinations: un-manipulated control plants, plants subject to tissue loss only, plants subject to jasmonate only, and plants subject to tissue loss and jasmonate together.

Our experiment used females of the wind-pollinated annual dioecious plant *Mercurialis annua* that have evolved greatly enhanced “leaky” sex expression following the experimental removal of all males from their populations, as a result of selection for reproductive assurance and greater siring success (Cossard and Pannell, 2021; Cossard et al., 2021). Leaky sex expression (the production of gametes of the opposite sex by plants with separate sexes) is common in dioecious plants and likely plays an important role in reversions from dioecy to hermaphroditism (Ehlers and Bataillon, 2007; Cossard and Pannell, 2019). Because the genotypes used in our experiment are females that are now expressing a greatly enhanced leaky male-flower production that has evolved in the absence of herbivory, we had no a priori expectation for how they should respond to simulated herbivory. However, previous work on dioecious *M. annua* has shown that simulated herbivory enhances leaky sex expression in both males and females (Villamil et al., 2021), for reasons that may or may not be adaptive. We might thus have expected this tendency for greater leakiness under herbivory to have been retained or even enhanced in the genotypes we used in our experiment. Surprisingly, we found that females with leakiness enhanced through experimental evolution actually reduced their male flower production in response to tissue loss in our experiment, a response that was erased in plants that were exposed to hormone signaling by jasmonates. Our experiment thus reveals complex norms of reaction to herbivory that cannot be adaptive (because they are expressed by newly evolved genotypes), but that represent intrinsic pleiotropic responses to prior selection on reproductive allocation.

### Materials and methods

#### Study system

*Mercurialis annua* (Euphorbiaceae) is an annual wind-pollinated herb distributed throughout central and western Europe and around the Mediterranean Basin (Tutin et al., 1968). The species has long been used as a model system to investigate the evolution of dioecy and sex expression (Yampolsky, 1919, 1930; Pannell, 1997b; Obbard et al., 2006) due to its great diversity and plasticity in sex expression and sexual systems (Yampolsky, 1930; Pannell, 1997a; Cossard and Pannell, 2021; Cossard et al., 2021). *M. annua* has chromosomal sex determination (XX♀; XY♂) (Russell and Pannell, 2015), mediated by endogenous hormonal signaling. Males are feminized by exogenous application of cytokinins, and females are masculinized by auxins (Durand and Durand, 1991). The plants used in this study are monoecious XX females that have recently evolved pollen production under natural selection under experimental evolution (Cossard et al., 2021) and are therefore now functionally hermaphroditic. These plants are an ideal system in which to test the effects of herbivory on sex allocation for two reasons. First, although *M. annua* has chromosomal sex determination, its sex expression is also hormonally regulated. Second, these plants have highly plastic sex allocation, which has evolved very recently; therefore, we expect the interference of defensive signals on sex allocation, if it occurs, to reflect deeply conserved physiological reaction norms that have not been shaped by recent natural selection. We are not aware of studies on the natural level of herbivory in wild populations of the diploid *M. annua*, but we know that in Western Europe its natural herbivores are slugs and snails (pers. obs.). Levels of natural herbivory are well-documented for the hexaploid populations of *M. annua*, which are androdioecious (populations in which males and hermaphrodites co-occur) and thus more similar in their sex allocation to the studied population. Herbivory is male-biased in the hexaploid *M. annua* populations, with males having damage levels two times greater than hermaphrodites (Sánchez-Vilas and Pannell, 2011), a consistent pattern amongst many plants with separate sexes (Cornelissen and Stiling, 2005).

#### Plant culturing

Seeds generated through the previous selection experiment (Cossard et al., 2021) were sown in plastic trays with sterilized soil (Ricoter 163 soil) and kept under stable greenhouse conditions (25°C, 50% humidity; October 2019) at all times. When seedlings had flushed their first four leaves, approximately 3 weeks after sowing, they were transplanted into individual
pots (Teku serie TO 14 D) with soil (Ricoter 140 soil) and slow-release fertilizer (Hauert Tardit: 3M 500 g for 100 L of soil). Plants were watered every 2 days by an automatic watering system.

Experimental design

Pots were haphazardly assigned to one of four experimental treatments: control, herbivory only, jasmonate only, and herbivory and jasmonate together. For plants under the control treatment (C), leaves were sprayed with a sham solution containing only water and polysorbate until all leaves were wet (see Appendix for detailed solution formulae). The herbivory treatment (H) consisted of cutting off half of every second leaf on the plant with scissors and spraying plants with a sham solution until all leaves were wet (defoliation resulted in a 25% reduction of total leaf area over the course of the whole plant’s lifetime). Leaves were trimmed by cutting them in half perpendicularly to their midrib using scissors. In the jasmonate treatment (IA), plants were sprayed with a solution of methyl-jasmonate (concentration of 0.01%) and polysorbate until all leaves were wet (polysorbate 20 was used to fix the methyl-jasmonate on the sprayed leaves). Finally, the jasmonate and herbivory treatment (IAH) consisted of cutting off half of every other leaf on the plant with scissors and spraying plants with the methyl-jasmonate solution until all leaves were wet. These treatments were applied repeatedly as plants continued to grow, i.e., they represent chronic stress and manipulation. The first round of treatment was applied 1 week after repotting the plants (25th of November 2019) and then every 2 weeks over the next 12 weeks (the last treatment was applied on the 2nd of February 2020). On the first round of treatment, when most plants had fewer than six leaves each, we cut off only half a leaf (~10% of the leaf area removed) for plants under the herbivory treatments to avoid seedlings death.

Because jasmonate is highly volatile and can be perceived through stomata by neighboring plants (Heil, 2009; Heil and Karban, 2010), plants from a given treatment were enclosed within two-meter curtains of transparent plastic to prevent “eavesdropping” cross-contamination. The eight identical enclosures consisted of an aluminum squared frame (130 cm × 130 cm) to which we attached four wooden poles (two meters long). The wooden structures were wrapped with 2 m-tall plastic curtains, which were fixed on three sides. The fourth side was held in place with pins acting as a door that allowed us to enter the enclosure for plant manipulations and measurements. To reduce the risk of contamination, enclosures remained closed at all times, and were opened only to allow access for measurements or manipulations. To avoid confounding effects of enclosure and treatment, we had a blocked design with two enclosures per treatment and 68 individuals per block.

Sampling

Plant sampling consisted of cutting all above-ground plant material of 34 plants per enclosure (N = 272) and recording total height. Plants were then cut in half, lengthwise, creating two distinct segments: top and bottom. The top segment was carefully examined and we counted the number of fruits (immature and mature) and harvested all male flowers using tweezers. Male flowers were stored in paper envelopes, dried and weighed. After phenotyping, plant segments were dried and weighed to obtain plant dry biomass (top + bottom). To estimate seed production, the seeds were isolated from the dried plant materials, stored in paper envelopes and weighed. All materials were dried in an oven at 50°C for at least 14 days and weighed using a digital scale.

To increase sampling efficiency, we first conducted a pilot subsampling study to test whether flower production on the top half of the plant was correlated with the total flower production of the whole plant. A strong correlation would allow considering the top plant segments as a good estimate (hereafter, subsample) of the whole plant. Plant subsampling consisted of cutting all above-ground plant material of five plants per enclosure (N = 40) and recording total height. The plants were cut in half, lengthwise, creating two distinct segments: top and bottom, both of which were phenotyped following the procedure detailed above. The accuracy of the top segment as a valid subsample was tested using a correlation between reproductive structures on the top segment versus reproductive structures on the whole plant (top + bottom segments). We found a significant and positive correlation (r = 0.71; P = 3.94−07) concluding that the top sections were an accurate representation of the whole plant flower production, and proceeded with phenotyping only the top segment of the remaining 232 plants. All further statistical analyses on reproductive effort were conducted considering only plant subsample data (top segment), even for those 40 plants which were entirely phenotyped.

Statistical analyses

All statistical analyses were conducted in R version 4.03 (R Core Team, 2021). Mixed-effect models were fitted using the “lmer4” package (Bates et al., 2015), residuals and model assumptions were checked using the “DHARMa” package (Hartig, 2022), and post-hoc Tukey comparisons were tested using the “multcomp” package (Hothorn et al., 2008), and variance explained by fixed and random factors was tested using the “MuMln” package (Barton and Barton, 2018). Model specifications, estimates and statistics are reported in Table 1. We included days-post-treatment (DPT) in our statistical models to account for the effects that the period elapsed between the last treatment application and the plant sampling date may have on our response variables. For logistical reasons, our
sampling was spread over 14 days by a team of six assistants. We included observer in our statistical analyses to account for possible differences among assistants.

To test the effects of herbivory on the male (MRE) and female (FRE) reproductive effort, defined as the proportion of biomass allocated to each sexual function per gram of biomass of the top subsample, we used Gaussian mixed models. To deal with zeroes and meet normality assumptions, we transformed MRE data by adding a value ten times smaller than the smallest non-zero value within its range, and applied the following formula: $\log(\text{MRE}) = \log(\text{MRE} + 10^{-6})$. Both of these models included as fixed effects treatment, DPT, and the biomass of the bottom plant section to account for the fact that larger plants invest more in reproduction; exclusion box (block) and observer were included as random effects.

We tested the effects of herbivory on plants’ investment in sexual reproduction using a Gaussian mixed model. Reproductive effort (RE), defined as the proportion of above-ground plant biomass allocated to sexual reproduction (including male and female flowers and fruits), was fitted as the response variable. Treatment, DPT, and the biomass of the bottom plant section were fitted as fixed effects; exclusion box (block) and observer were included as random effects. We tested whether plants responded to herbivory through compensatory growth using a Gaussian mixed model, fitting total above-ground plant biomass as the response variable, treatment and DPT as fixed effects, and the exclusion box (block) as a random effect (for this test, we did not include observer as a random effect because all samples were weighed by one person).

Results

Overall investment in the male function represented less than 3% of total aboveground biomass in hermaphrodites, four times less than the proportion of plant biomass invested in the female function (Figures 1A, B). Tissue loss significantly halved male reproductive effort in plants that suffered herbivory compared to control plants (C: 2.47 ± 0.47; H: 1.36 ± 0.25; mean ± SE; Figure 1A), but jasmonate application restored male reproductive investment, as shown by the lack of significant differences between JAH and control plants (Figure 1A and Table 1). Interestingly, jasmonate alone did not enhance investment toward the male function, as shown by the non-significant differences between control plants and those only sprayed with jasmonate (Figure 1A and Table 1). The time elapsed between the applications of the last treatment and sampling date (DPT) did not significantly affect male reproductive effort (Table 1).

Tissue loss significantly increased female reproductive effort in hermaphrodites by a quarter compared to control plants or those only sprayed by jasmonate (C: 9.22 ± 0.727; H: 12.58 ± 0.90; JA: 8.30 ± 0.69; mean ± SE; Figure 1B), but, again, jasmonate application in combination with tissue loss restored reproductive investment to levels similar to those of control plants, as shown by the lack of significant differences between JAH and control plants (Figure 1B and Table 1). The time elapsed between the application of the last treatment and sampling date (DPT) had a negative effect on female reproductive effort (Table 1). In all models, random effects explained very little of the variance of the response variables, indicating that the enclosure box and the observer had a negligible effect on our estimates (Table 1).

The herbivory treatments had no significant effect on plant biomass. Despite suffering 25% defoliation, plants under the herbivory treatment were on average only 8.85% lighter than control plants (C: 13.56 ± 0.46; H: 12.36 ± 0.44; mean ± SE; Figure 1D), a difference that was statistically non-significant (Figure 1D and Table 1). The time elapsed between the applications of the last treatment and sampling date (DPT) did not significantly affect plant biomass (Table 1). On average, approximately 25% of reproductive biomass was allocated to male flower production, whilst the remaining 75% was allocated toward the female function. However, neither tissue loss nor jasmonate application had a significant effect on the proportion of biomass allocated toward sexual reproduction (reproductive effort; Figure 1C and Table 1). Plants invested on average ~10% of their total above-ground biomass toward reproduction, even under chronic 25% defoliation. Similarly, jasmonate application had no significant effect on reproductive effort (Figure 1C). The time elapsed between the application of the last treatment and sampling date (DPT) had a negative effect on reproductive effort (Table 1).

Discussion

Even though chronic 25% defoliation had no significant effect on plant biomass, tissue loss significantly increased the proportion of biomass allocated toward reproduction. This increase in reproductive effort was invested toward the female function, at the expense of investment in the male function. However, exogenous jasmonate application effectively restored investment toward the male function in damaged plants.

Herbivory-induced sex allocation was altered by selection in the absence of herbivory

Our experiment revealed that tissue loss due to chronic simulated herbivory in M. annua caused females with enhanced leaky sex expression in response to recent selection (Cossard et al., 2021) to shift their sex allocation away from male flower production. This response was thus opposed to that of their less leaky recent ancestors, for which tissue damage increased male
flower production (Villamil et al., 2021). In other words, the very direction of the reaction norm of male flower production by females in response to tissue damage in natural populations of dioecious *M. annua* has been altered by strong selection on male flower production.

We do not know whether the reaction norm of male-flower production by females in the ancestral dioecious populations is adaptive, but it might be so (Villamil et al., 2021). However, we can safely say that the change in reaction norm that we have observed cannot be adaptive, for two reasons. First, females evolved enhanced male production in the selection experiment in the complete absence of sustained tissue damage (and more importantly in the absence of variation in tissue damage), so they were not exposed to selection that might have favored a different reaction norm. Second, the mean male flower production in the females of our experiment was much higher than is ever found in natural populations, such that, again, reaction norms around this new mean are not pertinent to the optimizing effects of natural selection in the ancestral population. The reaction norms we have measured are therefore clearly non-adaptive, and should properly be seen as a pleiotropic effect of selection on reproductive allocation.

Pleiotropic effects of natural selection have been found in selection experiments in a number of contexts (Hill and Caballero, 1992), including bacteria (Lenski, 1988a,b; Jasmin and Zeyl, 2013), insects (Harshman and Hoffmann, 2000), and plants (references cited below). A classic example is provided by Lenski's *Escherichia coli* lines, which evolved resistance to a virus along with maladaptive mutations on various metabolic pathways and which eventually resulted in reduced fitness levels, similar to those in non-resistant populations (Lenski, 1988a,b). A more recent example in *Drosophila* shows that evolved resistance against an intestinal pathogen evolved along with deleterious effects on male fitness through reduced male mating success and reduced sperm competitive abilities (Kawecki, 2020). In plants, maladaptive pleiotropic effects in response to an experimental selection pressure have been widely observed in the context of evolution of herbicide resistance (Bergelson et al., 1996; Debban et al., 2015), but also in that of the evolution of flower color (Coberly and Rausher, 2008), induced defenses (Siemens and Mitchell-Olds, 1998), flowering phenology (Kover et al., 2009), or increased plant size leading to decreased defensive strategies and reduced floral signals (Zu and Schiestl, 2017). Our study provides a further revealing illustration of the

### Table 1. Model outputs on the effects of herbivory on male reproductive effort, female reproductive effort, total reproductive effort, and plant biomass of *Mercurialis annua*.

| Predictors | Male reproductive effort | Female reproductive effort | Reproductive effort | Plant biomass |
|------------|---------------------------|----------------------------|---------------------|--------------|
|            | Estimates                  | p                          | Estimates           | p            | Estimates | p            | Estimates | p            |
| Treatment[C] | −5.740 (<0.001)           | 0.213 (<0.001)             | 0.226 (<0.001)      | 11.514 (<0.001) |
|            | (−8.187, −3.293)           | (0.157, 0.268)             | (0.175, 0.277)      | (7.983, 15.046) |
| Treatment[H] | −1.153 (0.048)            | 0.031 (0.042)              | 0.020 (0.083)       | −1.199 (0.341) |
|            | (−2.295, −0.010)           | (0.001, 0.060)             | (−0.003, 0.043)     | (−3.666, 1.268) |
| Treatment[IA] | −0.077 (0.895)            | −0.009 (0.559)             | 0.009 (0.433)       | 0.186 (0.882) |
|            | (−1.064, −1.218)           | (−0.038, 0.021)            | (−0.032, 0.014)     | (−2.281, 2.654) |
| Treatment[IAH] | −0.457 (0.432)           | 0.011 (0.473)              | 0.006 (0.629)       | −0.461 (0.714) |
|            | (−1.597, 0.684)           | (−0.019, 0.040)            | (−0.017, 0.029)     | (−2.928, −2.006) |
| DPT        | −0.012 (0.826)            | −0.004 (0.002)             | −0.004 (0.001)      | 0.100 (0.190) |
|            | (−0.0115, 0.092)          | (−0.006, −0.001)           | (−0.006, −0.001)    | (−0.049, 0.250) |
| Bottom biomass | 0.094 (0.062)           | −0.005 (<0.001)           | −0.005 (0.002)      | −0.006 (−0.001) |
|            | (−0.005, 0.194)           | (−0.007, −0.003)           | (−0.006, −0.001)    | 

### Random effects

| σ² | 7.36 | 0.00 | 0.00 | 15.97 |
|τ0 | 0.12 Box | 0.00 Box | 0.00 Box | 1.12 Box |
|ICC | 0.08 Observer | 0.00 Observer | 0.00 Observer | |
|N | 0.03 | 0.01 | 0.07 |
|6 | 0.8 Box | 0.8 Box | 0.8 Box | |
|Observations | 273 | 259 | 273 |
|Marginal R²/Conditional R² | 0.045/0.071 | 0.146/NA | 0.109/0.118 | 0.022/0.086 |

DPT, days-post-treatment; C, control; H, herbivory (25% chronic tissue loss); IA, jasmonate application; IAH, 25% chronic tissue loss and jasmonate application. Numbers in brackets show the upper and lower 95% CI of mean model estimates. Numbers in bold indicate significant differences (*P* < 0.05).
FIGURE 1

Direct (tissue loss) and indirect (jasmonate-mediated) effects of herbivory on (A) male reproductive effort (male inflorescence biomass/vegetative biomass), (B) female reproductive function (seed biomass/vegetative biomass), (C) total reproductive effort (reproductive biomass/vegetative biomass), (D) total aboveground plant biomass. Note that variables in panels (A–C) were calculated on the basis of measurements of the apical subsample for each individual sampled. Treatments are abbreviated as follows, C, control; H, herbivory treatment with a chronic tissue loss of 25% of foliar area; JA, jasmonate application; JAH, tissue loss and jasmonate application treatment. Different letters indicate significant differences between treatment levels ($P < 0.05$). Total $N = 273$ plants.

Tissue loss caused plants to reduce male sex allocation, but jasmonate signaling restored it

Our experiment tested both the direct (tissue loss) and indirect (jasmonate signaling) effects of herbivory on sex allocation in the evolved population of $M. \textit{annua}$. Interestingly, we found that plants reacted to both of these components differently. Male reproductive effort was nearly halved in defoliated plants, and jasmonate restored male investment after tissue loss, showing that signaling related to anti-herbivore defenses might also affect sexual expression. We expected the tissue loss and jasmonate application treatments to have additive effects on male investment, with the exogenous (jasmonate application) signal effectively amplifying the endogenous direct effects of tissue loss. However, jasmonate signaling caused plants to increase male net investment only if they had suffered tissue loss. To some extent, this result contrasts with previous findings showing that jasmonate can induce plant responses even in the absence of tissue loss (Heil et al., 2001a,b; Kost and Heil, 2008; Radhika et al., 2008; Kessler and Heil, 2011; Escalante-Pérez and Heil, 2012; Heil, 2015; Hernandez-Cumplido et al., 2016). If jasmonate on its
own enhanced male allocation, whilst tissue loss reduced it, we expected plants that only received additional jasmonate but suffered no tissue loss (JA) to have greater male investment than those under the tissue loss and jasmonate treatment (JAH). However, we found no differences between plants in JA and JAH treatments in male investment or any of the response variables measured. Our results thus show that an increased jasmonate signal perceived exogenously can restore male sex allocation only in damaged plants. As explained above, it is difficult to infer any functional or adaptive explanation for this result, but it is clear that different effects of damage interact and are not simply additive.

The exogenous application of jasmonate that restored male sex allocation to non-herbivory levels would be a favorably selected response in the recent evolutionary past of the *M. annua* population (in which males had been removed), because the male-flower production provides exceptionally high fitness through siring (Cossard and Pannell, 2021; Cossard et al., 2021). The ability of jasmonate to restore male sex allocation when plants are damaged may thus also be a pleiotropic effect of selection, perhaps as a result of a physiological constraint associated with hormonal "cross-talk." Clearly, with time and appropriate conditions, we should expect natural selection to act upon the reaction norms we have observed, gradually modifying the non-adaptive response to jasmonate in restoring male allocation and converting it to a response that could be seen as adaptive. For instance, this could occur if plants evolved increased sensitivity to jasmonate as a male-function restorer so that the smaller amounts of jasmonate released exclusively by damaged plants would suffice to restore their male sex allocation in defoliated plants. Such an evolved response would allow damaged females with enhanced leakiness (H) to maximize their fitness, regardless of the herbivory levels experienced by their neighbors, i.e., the additional jasmonate levels. Future research in other species with a longer evolutionary history under monocacy and under suitable variation in herbivory levels would be valuable to determine whether jasmonate is also capable of restoring tissue loss-induced shifts in sex expression and what its adaptive or deleterious consequences might be.

Our findings suggest that jasmonate signaling may connect defensive and reproductive strategies in *M. annua*. More specifically, they indicate that herbivory might affect sex expression through its direct effects on tissue loss, but not through its indirect influence on hormone signaling, as perceived from neighboring plants (by eavesdropping), and that jasmonate perceived in synergy with damage might restore the effects of tissue loss. Jasmonate has been found to play a role in plant responses to herbivory (Thaler et al., 2001; Heil and Bueno, 2007b; Heil and Karban, 2010; Ballaré, 2011) and in regulating sex expression, flower development and sexual differentiation in a number of other plants species (Acosta et al., 2009; Yan et al., 2012; Wasternack et al., 2013; Cai et al., 2014; Yuan and Zhang, 2015), and our results confirm that it can act upon both functions simultaneously in *M. annua*. To our knowledge, this is the first account of the influence of jasmonate on the sexual expression of a species in the Euphorbiaceae family. *M. annua* has chromosomal sex determination (Durand and Durand, 1991; Russell and Pannell, 2015), but its sex expression can be influenced and even switched via the exogenous application of phytohormones such as cytokinins and auxins (Durand and Durand, 1991). The pleiotropic effects of herbivory on sex expression observed in *M. annua* could well be due to hormonal “cross-talk” between jasmonate and sex-determining hormones such as cytokinins and auxins, which has been well-documented in other species (Robert-Seilaniantz et al., 2011; Naseem et al., 2015).

### Tissue loss increased female sex allocation

Chronic defoliation elicited a change in male-flower production in *M. annua* females, and this change was associated with a corresponding response in fruit production, through a sex-allocation trade-off. This pattern contrasts with the available evidence on monoeocious species in which defoliation had the opposite effect, i.e., reducing female, but not male allocation (Snyder, 1993; Quesada et al., 1995). Furthermore, our findings also contrast with previous studies in hermaphroditic species in which defoliation decreased male sex allocation, whilst female sex allocation remained unaffected (Allison, 1990; Frazee and Marquis, 1994; Lehtilä and Strauss, 1997). In this regard, our experimental population of monoeocious *M. annua* plants seems to have an herbivory-induced sex allocation response different from those previously reported in the literature for hermaphroditic or monoeocious species. As noted above, this unusual pattern may be attributable to the fact that it has not yet been molded by natural selection.

### No measurable negative impacts on performance and increased fitness: Evidence for tolerance?

Contrary to our expectations, tissue loss increased the proportion of biomass invested toward sexual reproduction. Despite a chronic 25% defoliation through the plants’ lifetime, we observed no trade-offs between growth and reproduction. Rather, we found that defoliated plants significantly increased their reproductive effort by 2% compared to control plants. Plants are known to respond to damage by herbivores in three main ways: by showing “resistance” (i.e., by investing in physical or chemical means to deter consumption); by “escaping” (i.e., evading attack through phenological mismatching); and/or through “tolerance” (i.e., compensating tissue loss with new...
growth) (Stamp, 2003; Boege et al., 2007; Fornoni, 2011). Tolerance can be considered the reaction norm of fitness in relation to a varying environment (i.e., damage), and although biomass is not a direct proxy of fitness, it can be related to fitness as a proxy of plant performance. Our experimental plants might have coped with damage through compensatory growth, given that we observed no measurable negative impacts of defoliation on performance (biomass) (Figure 1D). We conducted a power analysis using the R package “simr”; (Green and MacLeod, 2016) and found that, given our sample size (N = 272), our experiment had sufficient power (80% power) to detect effect sizes ≥ 25% on plant biomass, i.e., much greater than the effect sizes from our experiment (−8.5% and +1.3%). Thus, while it is possible that plants in our experiment compensated for lost tissue via a tolerance response, it lacked power to rule out substantial, non-compensatory, negative effects of herbivory.

Concluding remarks

Our experimental design did not allow us to avoid the release of jasmonate by plants that suffered tissue loss, since this volatile is released with plant damage. Logistical constraints precluded treatments such as wrapping half of the leaves in foil to prevent their photosynthetic function (an equivalent of tissue loss) but without releasing jasmonate volatiles. Such an approach might be suitable for experiments in which the treatment was applied once or only a few times, but it was not possible to carry this out for a sample of >200 plants treated every 2 weeks. Adding a procedural control to test that the jasmonate induction treatment worked would also be worthwhile; indeed determining the jasmonate concentration required for successful inductions would provide interesting information for adjusting the dose required to induce plant defense at different ontogenetic stages.

Despite the above caveats, our results indicate that tissue loss and jasmonate signaling both had cascading effects on sex allocation in M. annua, and that jasmonate can be involved in both sex expression and defense pathways simultaneously. Our results thus suggest that defense-related traits—such as those induced by jasmonate signaling—may link the evolution of reproductive and defensive traits, and that jasmonate may thus play an important role in conditional sex allocation in some plants. Conditional sex allocation provides an ideal theoretical framework to address knowledge gaps on the effects of plant defenses on trade-offs between male and female plant fitness and sex allocation, advancing our understanding on the role antagonists may have as selective agents shaping sex allocation trade-offs. Appropriate experimental evolution studies would be a valuable tool to test the potential of selection to optimize this response.

Data availability statement

The data presented in this study are deposited in the Zenodo repository: https://doi.org/10.5281/zenodo.5667348.

Author contributions

NV conceived the ideas and designed the experiment. BS and NV collected and analyzed the data. NV and JP led the writing of the manuscript with critical inputs from BS. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by funding from the Swiss National Science Foundation (grant 310030_185196).

Acknowledgments

We thank Aline Revel for assistance with growing plants and Juan Fornoni and Kai-Hsiu Chen for comments that improved earlier versions of this manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.
I. Variation in competitive fitness among mutants resistant to herbivory on internal ethylene production and sex expression in the wild radish, *Isomeris arborea*.

B. A. (2009). Pleiotropic effects of environment-specific adaptation in *Arabidopsis thaliana* for lima bean in nature. *J. Chem. Ecol.* 34, 1-13. doi: 10.1007/s10886-008-9494-0

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrika* 95, 31-43. doi: 10.1093/biomet/asn018

Karban, R., and Strauss, S. Y. (1992). Effects of herbivores on growth and reproduction of their perennial host *Ergonan plumus*. *Ecology* 74, 39-46. doi: 10.2307/1939449

Kawecki, T. J. (2020). Sexual selection reveals a cost of patterned resistance undetected in life-history assays. *Evolution* 74, 338-348. doi: 10.1111/evo.13895

Kessler, A., Halitschke, R., and Poveda, K. (2011). Herbivory-mediated pollinator limitation: negative impacts of induced volatiles on plant-pollinator interactions. *Ecology* 92, 1641-1649. doi: 10.1890/10.1143/389

Klinkhamer, P. G., De Jong, T. J., and Metz, H. (1997). Sex and size in cosexual reproduction of their perennial host *Erigeron glaucus*. *Annu. Rev. Ecol. Syst.* 28, 1363-1389. doi: 10.1146/annurev.es.23.110192.001443

Kost, C., and Heil, M. (2010). The defensive role of volatile emission and defence signalling: auxin and cytokinin modulate plant immune response pathways. *J. Exp. Bot.* 61, 4856-4865. doi: 10.1093/jxb/erq297

López, S., and Dominguez, C. (2003). Sex choice in plants: facultative adjustment of the sex ratio in the perennial herb *Begonia gracilis*. *J. Ecol.* 11, 1240-1252. doi: 10.1037/1041.003.03862x

Lucas-Barbosa, D. (2016). Integrating studies on plant–pollinator and plant–herbivore interactions. *Trends Plant Sci.* 21, 125-133. doi: 10.1016/j.tplants.2015.10.013

Manners, K., and Marquis, J. R. (2000). Fitness impacts of herbivory through indirect effects on plant–pollinator interactions in *Oenothera macrocarpa*. *Ecology* 81, 30-40. doi: 10.2307/1771311

Mumm, R., Schrank, K., Wegener, R., Schulz, S., and Hilker, M. (2003). Chemical analysis of volatiles emitted by *Pinus sylvestris* after induction by insect oviposition. *J. Chem. Ecol.* 29, 1235-1252. doi: 10.1023/A:1023841909199

Naseem, M., Kaldorf, M., and Dandekar, T. (2015). The nexus between growth and defence signalling: auxin and cytokinin modulate plant immune response pathways. *J. Exp. Bot.* 66, 4856–4865. doi: 10.1093/jxb/erv297

Nüené-Farfan, J., Cabrals-Vargas, R. A., and Durzo, R. (1996). Mating system consequences on resistance to herbivory and life history traits in *Datura stramonium*. *Am. J. Bot.* 83, 1041-1049. doi: 10.2307/29619361

Pannell, J. (1997b). Variation in sex ratios and sex allocation in androdioecious *Mercurialis annua*. *Heredity* 78, 50-56. doi: 10.1038/hdy.1997.6

Pannell, J. (1997). Mixed genetic and environmental sex determination in an androdioecious population of *Mercurialis annua*. *Heredity* 78, 50-56. doi: 10.1038/hdy.1997.6

Pannell, J., Dorken, M. E., Pusol, B., and Berjano, R. (2008). Gender variation and transitions between sexual systems in *Mercurialis annua* (Euphorbiaceae). *Int. J. Plant Sci.* 169, 129-139. doi: 10.1086/523360

Policansky, D. (1987). Sex choice and reproductive costs in jack-in-the-pulpit. *BioScience* 37, 476–481. doi: 10.2307/1309419

Poveda, K., Stefan-Dewenter, I., Scheu, S., and Tischmarche, T. (2003). Effects of below- and above-ground herbivores on plant growth, flower visitation and seed set. *Oecologia* 135, 601–605. doi: 10.1007/s00442-003-1228-1

Quesada, M., Bollman, K., and Stephenson, A. G. (1995). Leaf damage decreases pollen production and hinders pollen performance in *Cucurbita texana*. *Ecology* 76, 437–443. doi: 10.1890/017954

Ramos, S. E., and Schielz, E. (2019). Rapid plant evolution driven by the interaction of pollination and herbivory. *Science* 364, 193–196. doi: 10.1126/science.aav6962

Reynolds, R., and Farmer, E. H. (1989). Jasmonate and salicylate as global signals in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Plant Physiol. Biochem.* 40, 342–348. doi: 10.1111/j.1469-8137.2009.02943.x

Russell, J., and Pannell, J. (2015). Sex determination in dioecious *Mercurialis annua* and its close diploid and polyploid relatives. *Heredit* 114, 262–271. doi: 10.1038/hdy.2014.95

Salvador-Retallal, V., Tjallingii, W. F., and Farmer, E. E. (2014). Real-time, in vivo intracellular recordings of caterpillar-induced depolarization waves in sieve
elements using aphid electrodes. New Phytol. 203, 674–684. doi: 10.1111/nph.12807

Sánchez-Vilas, J., and Pannell, J. R. (2011). Sex-differential herbivory in androdioecious Mercurialis annua. PLoS One 6:e22083. doi: 10.1371/journal.pone.0022083

Santangelo, J. S., Thompson, K. A., and Johnson, M. T. (2011). Sex-differential herbivory in androdioecious Mercurialis annua. PLoS One 6:e22083. doi: 10.1371/journal.pone.0022083

Siemens, D. H., and Mitchell-Olds, T. (1998). Evolution of pest-induced defenses in Brassica plants: tests of theory. Ecology 79, 632–646. doi: 10.1890/0012-9658(1998)0632:EOPIED2.0.CO;2

Stamp, N. (2003). Out of the quagmire of plant defense hypotheses. Q. Rev. Biol. 78, 23–55. doi: 10.1086/367580

Villamil, N., Li, X., Seddon, E., and Pannell, J. (2021). Herbivory enhances leaky sex expression in the dioecious herb Mercurialis annua. Ann. Bot. 129, 79–86. doi: 10.1093/aob/mcab129

Villamil, N., Li, X., Seddon, E., and Pannell, J. (2021). Herbivory enhances leaky sex expression in the dioecious herb Mercurialis annua. Ann. Bot. 129, 79–86. doi: 10.1093/aob/mcab129

Wang, J., Wu, D., Wang, Y., and Xie, D. (2019). Jasmonate action in plant defense against insects. J. Exp. Bot. 70, 3391–3408. doi: 10.1093/jxb/erz174

Wang, Y., Luo, A., Lyu, T., Dimitrov, D., Xu, X., Freckleton, R. P., et al. (2021). Global distribution and evolutionary transitions of angiosperm sexual systems. Ecol. Lett. 24, 1835–1847. doi: 10.1111/ele.13815

West, S. (2009). Sex Allocation. Princeton, NJ: Princeton University Press. doi: 10.1515/9781400832019

Yampolsky, C. (1919). Inheritance of sex in Mercurialis annua. Am. J. Bot. 6, 410–442. doi: 10.1002/j.1537-2197.1919.tb05554.x

Yampolsky, C. (1930). Induced alteration of sex in the male plant of Mercurialis annua. Bull. Torrey Bot. Club 57, 51–58. doi: 10.2307/24809510

Yan, Y., Christensen, S., Isakett, T., Engelberth, J., Meoley, R., Hayward, A., et al. (2012). Disruption of OPRI and OPRII reveals the versatile functions of jasmonic acid in maize development and defense. Plant Cell 24, 1420–1436. doi: 10.1085/jcb.111.094151

Yuan, Z., and Zhang, D. (2015). Roles of jasmonate signalling in plant inflorescence and flower development. Curr. Opin. Plant Biol. 27, 44–51. doi: 10.1016/j.pbi.2015.05.024

Zu, P., and Schiestl, F. P. (2017). The effects of becoming taller: direct and pleiotropic effects of artificial selection on plant height in Brassica rapa. Plant J. 89, 1009–1019. doi: 10.1111/tpj.13440
Appendix

Appendix 1: Protocol for preparing sham and methyl-jasmonate solutions

Methyl-jasmonate solution:

1. 1 mL of Polysorbate 20 (tween20) gauged to 250 mL of distilled water.
2. 0.1 mL of Methyl-jasmonate gauged to 250 mL of distilled water.
3. Mix both solution, gauge to 1,000 mL and transfer to a jar with an airtight lid.
4. Vortex the mix 45 s (closed jar to avoid volatiles escaping) and keep the solution at 4°C.

Sham solution used for the control treatment.

1. 1 mL of Polysorbate 20 (tween20) gauged to 1,000 mL of distilled water.
2. Transfer to a jar with an airtight lid.
3. Vortex the mix 45 s (closed jar) and keep the solution at 4°C.