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Sex expression and the selection and evolution of combined sexes in the dioecious annual herb *Mercurialis annua*

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par

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Prof. Mehdi Tafti

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Abstract

The flowering plants display a large variety of sexual systems that has long astonished botanists. The great majority of species are hermaphroditic, carrying male and female organs within the same flower, while others are characterized by sexes separated between different individuals, a system termed dioecy. The later is likely to have evolved from the ancestral state of hermaphroditism and had long been considered an evolutionary dead-end. However recent progress in our understanding of transitions between sexual systems in plants has shed light on frequent transitions away from dioecy, arguing against the evolutionary dead-end hypothesis. The determinants of transitions towards dioecy likely involve an intermediate step of either gynodioecy (where females coexist with hermaphrodites) or monoecy (where individuals carry unisexual flowers of both sexes). The principle aim of this thesis was to investigate factors that may lead dioecious species towards a state of combined sexes, either hermaphroditism or monoecy. My investigation builds on seminal work conducted by the evolutionary theorist David Lloyd in the 1970s, who observed the often labile nature of sex expression in dioecious populations, where females and males may sometimes produce pollen or seeds, respectively. Lloyd proposed that this ‘inconstancy’ or ‘leakiness’ in sex expression constitutes variation on which natural selection can act to bring about the breakdown of dioecy and the evolution of combined sexes. To investigate this idea, I studied populations of *Mercurialis ann*ua, a dioecious herb growing in Europe and around the Mediterranean Basin and that is characterized by the presence of lability in sex expression. My goals were to assess how males and females differ, both in their patterns of inconstancy and the underlying gene expression profiles, and to determine the functionality of this inconstancy in natural dioecious populations. I found that most of the inconstant reproductive effort of both males and females had a negligible effect on fitness in natural conditions. However, I reasoned that when plants are isolated from large populations, inconstant individuals should enjoy a strong advantage by being able to reproduce by selfing. Because I detected that females were more frequently inconstant than males in natural populations, I hypothesized that females should benefit more from isolation. I tested this hypothesis by removing males from experimental populations of dioecious *M. annua* and allowing the remaining females to evolve in their absence. These females showed a dramatic increase in pollen production over four generations, with a large proportion of the male-less populations being functionally hermaphroditic. I also investigated how the profiles of sex expression in the evolved individuals had shifted, and found that most of the genes that were originally more highly expressed in males than females to be up-regulated as well in the selected females. Together, my results show that inconstancy is a trait underlined by genetic variation on which selection may act during episodes of isolation, and can indeed provide a starting point for transitions away from dioecy.

Résumé

La grande diversité de systèmes sexuels qui présentent les plantes à fleurs à depuis longtemps intrigué les botanistes. La grande majorité des espèces sont hermaphrodites, portant les organes mâles et femelles au sein de la même fleur, tandis qu'une minorité se caractérise par la séparation des sexes entre différents individus, un système appelé dioécie. Ce-dernier a probablement évolué à partir de l'état ancestral hermaphrodite et a longtemps été considéré comme une impasse évolutive. Cependant de récentes avancées dans notre compréhension de l'évolution des transitions entre systèmes sexuels chez les plantes semblent indiquer que la dioécie peut évoluer vers des systèmes où les sexes sont combinés, contredisant l'hypothèse de l'impasse évolutive. Les déterminants des transitions vers la dioécie impliquent sûrement des étapes intermédiaires de gynodioécie (où femelles et hermaphrodites coexistent) ou de monoécie (où les individus portent des fleurs unisexuelles des deux sexes). Le but principal de cette thèse est d'étudier les facteurs qui peuvent amener une espèce dioïque a transiter vers un état de sexes combinés, soit l'hermaphroditisme, soit la monoécie. Mon étude se base sur le travail fondateur du théoricien David Lloyd dans les années 1970, qui a observé que la nature souvent labile de l'expression du sexe dans les populations dioïques, où les femelles et les mâles parfois produisent du pollen et des graines, respectivement. Lloyd a proposé que cette 'inconstance' dans l'expression du sexe pourrait constituer un variation sur laquelle la sélection pourrait agir et déclencher une rupture de la dioécie et l'évolution d'un système où les sexes sont combinés. Afin d'explorer cette idée, j'ai étudié les populations de *Mercurialis annua*, une herbe dioïque présente en Europe et autour du bassin méditerranéen, caractérisée par la présence d'inconstance de l'expression du sexe. Mes objectifs étaient d'estimer la divergence entre les mâles et les femelles, à la fois dans la façon dont ils expriment l'inconstance du sexe et dans leur profiles d'expression de gènes, et de déterminer la fonctionnalité de l'inconstance dans les populations naturelles. J'ai trouvé que la majeure partie des organes sexuels produits par inconstance n'avaient qu'un effet négligeable sur le succès reproducteur d'un individu. Cependant il apparaît que lorsque les plantes se retrouvent isolées des larges populations, les individus inconstant devraient bénéficier d'un fort avantage en étant capables de se reproduire par autofécondation. Parce que j'ai détecté que les femelles étaient plus fréquemment inconstantes que les mâles dans les populations naturelles, j'ai émis l'hypothèse que les femelles devraient être particulièrement avantagées en cas d'isolement. J'ai testé cette hypothèse en constituant des populations expérimentales de *M. annua* dioïque desquelles j'ai supprimé les mâles, en laissant les femelles évoluer en leur absence. Ces femelles ont montré une importante augmentation de leur production de pollen en quatre générations, avec une large fraction de ces populations expérimentales étant effectivement fonctionnellement hermaphrodites. J'ai aussi étudié la façon dont les profiles d'expression de gènes qui différenciaient les mâles et les femelles dans les populations naturelles ont évolués chez durant l'expérience. Je montre que la plupart des gènes qui étaient naturellement plus fortement exprimés chez les mâles que les femelles avaient été surexprimés chez les femelles évoluées. Mes résultats suggèrent que l'inconstance du sexe est un trait soutenu par une variation génétique sur laquelle la sélection peut effectivement agir durant des épisodes d'isolement et peut, en effet, fournir un point de départ à des transitions depuis un état dioïque vers un état de sexes combinés.

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General introduction

Since their ancestors diverged from the Gymnosperm lineage in early-middle Jurassic, some 215 to 275 million years ago (Magallón, 2010), flowering plants, or angiosperms, have successfully colonized most of the terrestrial ecosystems and diversified into an estimated 250,000 to 300,000 species (Wikström *et al.*, 2001). Angiosperm species display an extraordinary diversity of forms of flowers, which evolved to optimize the transfer of pollen to ovules among or within flowers through the interplay of various vectors (Barrett, 2010). Around 90% of angiosperm species are hermaphroditic, i.e., both male and female functions are combined within the same 'perfect' bisexual flower. A significant number of species deviate from this common pattern by displaying various degrees of separation of male and female functions between flowers (dicliny) and between individuals (dimorphy). In terms of allocation to male versus female functions (sex allocation), the most contrasting sexual system is dioecy, where sexual functions are fully separated between male and female individuals (Figure 1).

The extreme diversity of sexual systems reflects frequent evolutionary transitions between different reproductive strategies. This thesis describes several related studies that aim to throw new light on one particularly poorly understood evolutionary path, namely that from separate sexes (dioecy) to combined sexes (hermaphroditism). In this introductory chapter, I provide an overview of what is known about the sexual system diversity, transitions between sexual systems, and sexual conflicts in the flowering plants. This background knowledge sets the stage for the questions I address in the chapters that follow, which I outline further below. Finally, I give a brief description of the model system I have adopted to address the thesis' aims and objectives.

Hermaphroditism is ancestral in angiosperms (Renner & Ricklefs, 1995; Renner, 2014), so that sexes primarily evolved from being combined in single flowers to becoming separated. This observation has led researchers to consider hermaphroditism and dioecy as two ends of a continuum in the distribution of sexual functions in plant populations. In his work on the diversity of plant sexual systems, Darwin (1877) was puzzled about 'why hermaphrodite plants should ever have been rendered dioecious' (p. 279). Current theorizing suggests that the answer may lie both in the advantage of avoiding selfing, which brings about the deleterious effects of inbreeding depression (Darwin, 1876), and in the advantages of becoming specialized in one of the two sexual functions (Charnov *et al.*, 1976; Charnov, 1979; Bawa, 1980; Givnish, 1980; Bawa & Beach, 1981). Discussion over which of these two advantages predominates in nature is still ongoing, but the answer will probably vary from one case to another (Freeman *et al.*, 1997).

A recent assessment, based on phylogenetic reconstruction, estimates that separate sexes have originated independently between 871 and 5,000 times among 38% to 43% of angiosperm families (Renner, 2014), by a variety of evolutionary pathways. Each of these pathways likely represents a singular evolutionary path that has led to a species-specific pattern of sex expression and sex determination (Beukeboom & Perrin, 2014). This variety of systems makes it difficult to identify the forces that have led to the evolution of separate sexes. However, common patterns are distinguishable when comparing among dioecious species (Lloyd, 1980a), or comparing dioecious species with their close relatives (Yampolsky & Yampolsky, 1922; Weiblen *et al.*, 2000; Renner, 2014).

Pathways to dioecy

The emergence of dioecy in a given lineage is thought to have followed one of three main pathways that differ in the way sexes are determined, contrasted, and constrained: the dimorphic pathway, the monomorphic pathway, and the direct pathway (Charlesworth & Charlesworth, 1978a,b; Lloyd, 1980; Dellaporta & Calderon-Urrea, 1993; Barrett, 2002; Delph, 2009).

The dimorphic pathway

Intuitively, the evolution of dioecy from hermaphroditism requires the suppression of male function in females and female function in males (Darwin, 1877). It seems unlikely that these two events should occur simultaneously in a population. Models for the evolution of separate sexes have thus sought conditions that might permit male- and female-sterility mutations to spread sequentially in a hermaphroditic population, creating intermediate steps of either gynodioecy (where females coexists with hermaphrodites) or androdioecy (where males and hermaphrodites coexist) (Figure 1A). These models point to selfing and the avoidance of inbreeding depression as the major driving force in the evolution of dioecy, so that the spread of sterility mutation depends on the selfing rate of the population, s, as well as on the level of inbreeding depression, δ. In addition, individuals that become sterile for one function might compensate the loss by increased allocation, $1 + k$, to the remaining sexual function, a phenomenon Darwin (1877) termed ‘compensation’, i.e., a trade-off in resource allocation between sexual functions. Charlesworth & Charlesworth (1978a) demonstrated that a male-sterility mutation can invade a hermaphroditic population only if $1 + k > 2(1 - s\delta)$. This both means that, in fully outcrossing populations ($s = 0$) or when there is no inbreeding depression, females can invade when $k > 1$ (i.e., when females produce twice the number of gametes produced by hermaphrodites), and that, even in the absence of compensation, females should be able to invade when $s\delta > 0.5$, i.e., when there exists substantial selfing and strong inbreeding depression.

In the case where a male-sterility mutation arises in the cytoplasmic genome (usually the mitochondrion), the sex of the progeny is maternally inherited so that male-sterile individuals will invade if they display the slightest increase in seed production compared to hermaphrodites (Lloyd, 1974; Figure 1A). Typically, cytoplasmic male-sterility will give rise to gynodioecious populations with a high female frequency when a nuclear restorer does invade a population (Case & Caruso, 2010). In general this type of sterility is the result of complex nucleo-cytoplasmic interaction (Charlesworth & Ganders, 1979). Models show that nucleo-cytoplasmic interactions that usually lead to stable gynodioecy (Gouyon *et al.*, 1991) may also select for maleness in gynodioecious populations, under the control of different nuclear restorer alleles, and can lead to either dioecy or trioecy (a polymorphism where males, females and hermaphordites co-exist), depending on the ability of canalized males to fully replace hermaphrodites (Maurice *et al.*, 1994). Under these conditions, the evolution of gynodioecy and dioecy is permitted in a wider range of conditions than in the case of pure nuclear determination of sex allocation, at the expense of hermaphroditism and androdioecy (Maurice *et al.*, 1994).

The conditions for the initial spread of female-sterility mutation are more stringent than in the case of male-sterility, and require that $1 + k > 2(1 - s\delta) / (1 - s)$. Indeed, the propensity for female-sterile individuals to pass on their genes to the next generation is ultimately limited by the number of available ovules to be sired in a population. In a fully outcrossing population, males must sire twice as many seeds as hermaphrodites ($1 + k > 2$) to be able to invade. However, the higher the level of selfing in a population, the lower the availability of ovules for obligately outcrossing males, so that selfing by hermaphrodites tends to prevent the spread of female-sterility mutations in their midst. Cytoplasmic female sterility cannot usually be transmitted to progeny, with the exceptions of angiosperm species characterized by biparental or paternal organelle inheritance (Reboud & Zeyl, 1994; Mogensen, 1996), so it cannot promote the emergence and maintenance of androdioecy. A gynodioecious state is thus more likely to precede dioecy than an androdioecious one (Charlesworth & Charlesworth, 1978a; Charlesworth, 1984), which seems in accordance with the extreme rareness of androdioecy in flowering plants (Charlesworth, 1984; Liston *et al.*, 1990; Renner & Ricklefs, 1995; Dommée *et al.*, 1999; Pannell, 2002; Renner, 2014), and the frequent association of gynodioecy and dioecy within angiosperm families (Dufay *et al.*, 2014). After the establishment of a gynodioecious population, the second step may occur by the subsequent spread of dominant female-sterility mutations among hermaphrodites, or via frequency-dependent selection on their male allocation (Charlesworth & Charlesworth, 1978a), i.e., sexual specialization towards greater male function in the remaining hermaphrodites. In the gynodioecy pathway, both the effects of

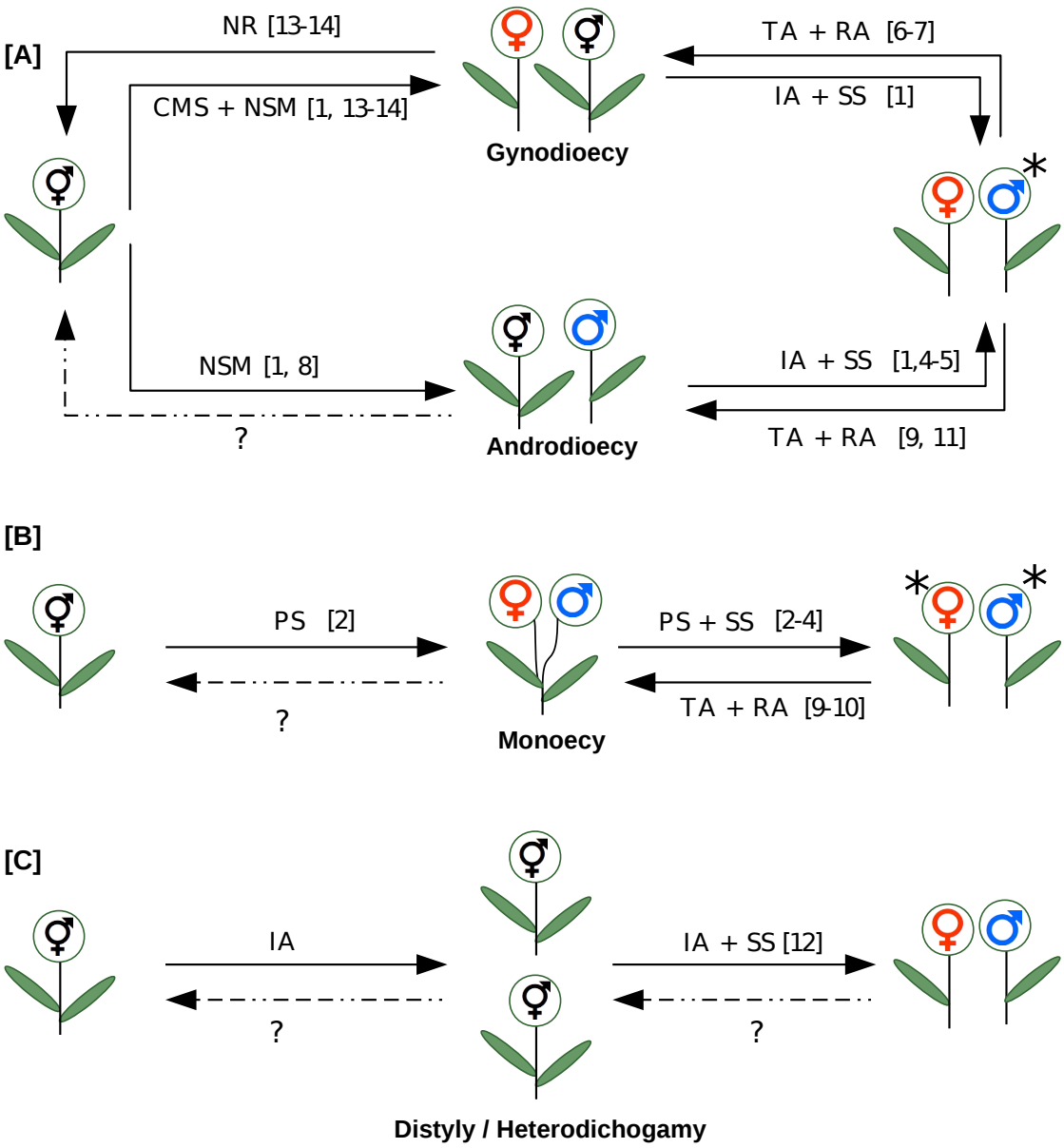

Figure 1. Schematic representation of inferred transitions between sexual systems in flowering plants along the three evolutionary pathways. **(A)** Dimorphic pathway, **(B)** monomorphic pathway, **(C)** Direct pathway. Stars indicate inconstant sex expression in dioecious individuals. Arrows indicate the direction of transitions between sexual systems. Dashed-arrows show transitions that have not been modeled yet. **NSM**: nuclear sterility mutation; **CSM**: cytoplasmic sterility mutation; **PS**: partial male-sterility; **NR**: nuclear restorer; **IA**: inbreeding avoidance; **SS**: sexual specialization; **TA**: transmission advantage; **RA**: reproductive assurance. References to the main modeling studies describing transitions are indicated next to arrows. [1] Charlesworth and Charlesworth, 1978a; [2] Charlesworth and Charlesworth, 1978b; [3] Charnov, 1976; [4] Charnov, 1982; [5] Charleworth, 1984; [6] Crossman & Charlesworth, 2014; [7]; Ehlers and Bataillon, 2007; [8] Billiard *et al.*, 2015; [9] Pannell, 2000; [10] Lloyd, 1975; [11] Wolf and Takebayashi, 2004; [12] Pannell and Verdu, 2006; [13] Charlesworth & Ganders, 1979; [14] Bailey *et al.*, 2003.

inbreeding avoidance and sexual specialization, acting sequentially during the transition, are probably responsible for the separation of sexes (Freeman *et al.*, 1997).

The monomorphic pathway

Alternatively, in the monomorphic pathway, monoecy is considered an intermediate step in the evolution of separate sexes. Monoecious species, characterized by the production of unisexual flowers, are known to be able to alter their floral sex ratios as the environment varies, allowing them to maximize their fitness (Freeman *et al.*, 1981; Silvertown, 1987; Charlesworth & Morgan, 1991). The flexibility of sex allocation of monoecy compared to hermaphroditism has been hypothesized to be responsible for its evolution (Charnov & Bull, 1977). Adaptive variation of the floral sex ratio in response to environmental variation has been observed in many monoecious species (e.g., Charlesworth & Charlesworth, 1981; Freeman *et al.*, 1981; McKone & Tonkyn, 1986; Costich, 1995; Sarkissian *et al.*, 2001), suggesting underlying genetic variation and the possibility of selection towards separate sexes (Charnov, 1982). In addition, a biased sex allocation in monoecious individuals may result in lower selfing rates, favouring the invasion by unisexuals. Monoecy is shared by about 6~7% of angiosperm species, and is disproportionately found associated with dioecy in angiosperm families, particularly in woody plants (Renner & Ricklefs, 1995; Renner, 2014). The gradual optimization of sex allocation from the sole action of sexual specialization, by which initially hermaphroditic individuals progressively enhance their efficiency at reproducing via one sexual function, while progressively losing the other one, may eventually lead to functional dioecy, too (Figure 1b).

In his seminal work on sex allocation, Charnov (1982) proposed a theoretical framework that models quantitatively the conditions for the evolution of sexual specialization or, in contrast, for allocation to both sexual functions simultaneously. In particular, Charnov *et al.* (1976) introduced the use of fitness gain curves, i.e., the relationship between an individual's reproductive success and its relative investment to male versus female functions. Such an approach leads to the prediction that dioecy should be positively selected when the rate of increase in fitness through one sexual function increases with the amount of resources allocated in that sex, i.e., accelerating fitness returns. In contrast, hermaphroditism should be the evolutionarily stable strategy (ESS) when the fitness gain curves flatten off with investment. Dioecious species that have followed this pathway are fundamentally constituted of "monoecious individuals that have reallocated their resources into one function or the other" (Charnov *et al.*, 1976).

The direct pathway

Finally, a direct transition from hermaphroditism to separate sexes was proposed by Darwin in his work on plant sexual systems (1877). He stated that separate sexes might emerge through the progressive separation of the 'two bodies of individuals in approximately equal numbers' (p. 284), which constitute populations of distylous or heterodichogamous species in which sexes are spatially or temporarily separated, respectively (Darwin, 1877; Lloyd & Webb, 1986; Pannell & Verdú, 2006; Gleiser *et al.*, 2008). So far, few examples support this hypothesis, although it has been inferred in some species (Pailler *et al.*, 1998; Pannell & Verdú, 2006; Rosas & Domínguez, 2009).

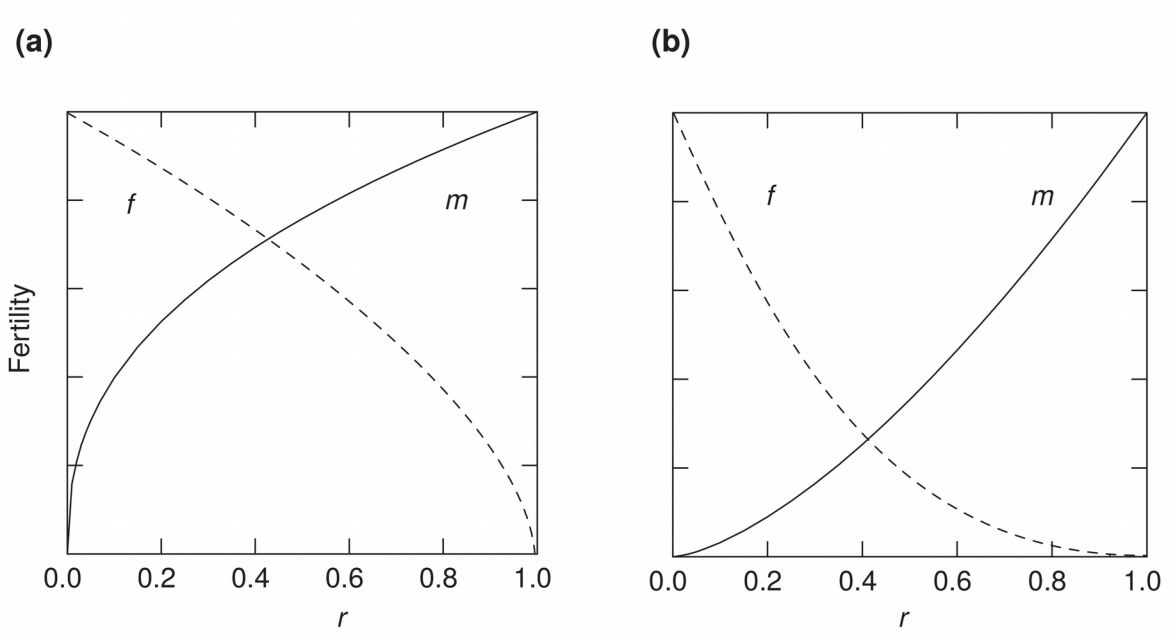

Figure 2. Fitness-gain curves, representing fertility as a function of the proportion of resources invested in male functions, *r.* (modified from Campbell, 2000). The value of *r* that maximizes the sum of the fitness derived from male (*m*) and female (*f*) investments corresponds to the ESS of sex allocation (Charnov 1982). **(a)** Concave gain curves showing diminishing returns with investment will favor a hermaphroditic strategy. **(b)** Convex gain curves showing increasing returns with investment will favor a strategy with separate sexes. Note that male and female gain curve often have divergent shape so that optimal sex allocation may often be biased in the direction of the sex showing the highest fitness returns.

Evolutionary implications of dioecy: sex-specific selection

We have previously discussed factors that can be responsible for the evolution of separate sexes under disruptive selection. Whether due to inbreeding avoidance, the advantages of sexual specialization or a combination of both, the evolution of dioecy implies diverging selective forces driving sexes away from each other, i.e., sex-specific selection.

Sex-specific selection arises when mutations have diverging fitness effects between sexes. First, a mutation can be deleterious in one gender but almost neutral in the other, so that the former will experience higher level of purifying selection. Such deleterious mutations seem to often be more detrimental to male fitness than female fitness, as has been recorded in a number of studies in *Drosophila melanogaster* (Sharp & Agrawal, 2008, 2013; Hollis *et al.*, 2009; Mallet *et al.*, 2011). Empirical studies show that sexual selection should accelerate the purging of such deleterious mutations in both sexes, with consequences on population growth. In the case where a mutation is deleterious for one sex only, these are hidden from purifying selection in half of the individuals, which is expected to allow their accumulation and increase the genetic load of a population (Connallon & Clark, 2010; Connallon & Jordan, 2016).

Alternatively, a mutation can be beneficial to one sex but deleterious to the other, a situation termed 'sexual antagonism' (SA), i.e., where selection acts on a single locus in opposing directions between the sexes. Models show that in species with separate sexes, SA is almost inevitable when directional selection or the phenotypic effects of mutations differ between the sexes (Connallon & Clark, 2014). SA selection seems to be particularly accentuated by the differing patterns of sexual selection between sexes in nature (Cox & Calsbeek, 2009). Indeed, according to Bateman's principle (Bateman, 1948), fitness gain through the resource-demanding female function is likely to be limited by resource availability, whereas male reproductive success depends primarily on mate availability. As a result, sexual selection is expected to be higher in males than in females (Bateman, 1948; Charnov, 1979; Arnold, 1994; Delph *et al.*, 2005; Delph & Ashman, 2006; Moore & Pannell, 2011), whether through male-male competition or female-choice (Stephenson and Bertin, 1983).

SA selection results in sexual conflicts within the genome, and leads to divergent patterns of genetic diversity between sexes, in turn influencing their evolutionary potential and that of the whole population (Meagher, 1994). This represents a particular form of sexual conflict, namely 'intra-locus sexual conflicts', where shared genes are the focus of sex-specific selection. SA selection is expected to lead to increased polymorphism at SA loci, responsible for increased fitness variance in the population (Patten *et al.*, 2010), and to the evolution of linkage disequilibrium (LD) between them (Patten *et al.*, 2010). These predictions are supported by empirical evidence in plants: artificial selection has revealed that sexually dimorphic reproductive traits in *Silene latifolia* are mostly underlined by quantitative trait loci (QTL), which seem to be present in greater number in males than females (Delph *et al.*, 2010). Moreover, most of these QTL were expressed only in one sex, suggesting that they evolved because of sexual conflicts. Recent modeling studies further indicate that increased LD among SA loci, known to lower the efficacy of natural selection (Hill &

Robertson, 1966), may facilitate the accumulation of deleterious mutations near them (Connallon & Jordan, 2016), which in turn affect the strength of inbreeding depression.

Finally there might exist sex-specific selection acting on males for increased mutation rates compared to females. Males are thought to be selected *via* sexual selection to produce a large number of gametes, with a greater rate of meiotic cell division during gametogenesis responsible for an increased mutation rate (Whitlock & Agrawal, 2009). These predictions appear to be supported by data among vertebrate species (Bartosch-Härlid *et al.*, 2003), as well as in plants like *Silene dioica*, in which higher mutations rates have been detected on Y-linked genes compared to the X-chromosome (Filatov & Charlesworth, 2002).

Evolutionary implications of dioecy: sexual dimorphism

When sexual functions are separated during the evolution of dioecy, males and females often evolve diverging phenotypes, probably in order to adapt to their specific sexual function. The phenotypic divergence of sexes, termed sexual dimorphism, is thus usually presented as a consequence of the divergence of sex-specific optima, i.e., a different trait value maximizing fitness in males versus females In dioecious plants, such cases of sexual specialization can be manifest either in the increased production of gametes of the specialized sex, and/or in the development of traits that enhance reproductive success through a particular sexual function. In the later case, males and females diverge not only in primary sexual functions, but also in morphological or physiological traits indirectly related to reproduction (Geber *et al.*, 1999). These characters are gathered under the term ‘secondary sexual characters’, and can be strongly dimorphic between the sexes (reviewed in Geber et al., 1999; Barrett & Hough, 2012).

Pollen production and dispersal is likely to depend on different resources than that needed for seed maturation and dispersion. For instance, in some wind-pollinated plants, males allocate large amounts of nitrogen to their pollen, whereas carbon is the main resource invested by females in seed maturation (Delph *et al.*, 1993; Harris & Pannell, 2008). Trade-offs of resource allocation between growth and reproduction might thus differ between sexes, especially in resource-poor environments, which can enhance the degree of divergence between sexes (Bazzaz *et al.*, 2000). Such sexual conflicts occurring after mating are thought to result from diverging ‘somatic costs of reproduction’ between sexes. As a consequence the level of sexual dimorphism observed in a species is expected to be higher at sexual maturity, in particular after fertilization has occurred.

On the other hand sexes can show divergent interests before reproduction so that phenotypic sexual dimorphism may be observable prior to flowering, as is sometimes observed in plants (reviewed in Barrett & Hough, 2012). Such pre-reproductive sexual dimorphism is thought mainly to be underlined by sexual selection in nature (Arnqvist & Rowe, 2005). Mating in plants occurs through the interplay of pollen vectors, so that we might expect pre-mating selection to favor improved use of these vectors (e.g., Fenster *et al*., 2004; Friedman & Barrett, 2009). The development of traits involved in gamete production and dispersion of course need to occur prior to mating, so that pre-mating sexual conflicts driven by sexual selection can be present in plant populations at the earliest stages of plant development. We currently lack information for plants on whether the phenotypic consequences of sexual conflicts will affect traits expressed only after mating has occurred, mainly resulting from differing costs of reproduction, or whether pre-mating development can also be affected and selected to adapt to sex-specific optima long before flowering.

Because males and females share the same genome, the evolution of sexual dimorphism under sex-specific selection may often lead to intra-locus sexual conflict, as noted above (Griffin *et al.*, 2013). The resolution of these conflicts is thought to permit the evolution of SD (Ellegren & Parsch, 2007; Bonduriansky & Chenoweth, 2009; Parsch & Ellegren, 2013). A number of mechanisms can account for the relaxation of these constraints and the evolution of SD, namely gene duplications, the evolution of sex chromosomes and sex-biased gene expression, and genomic imprinting. First, under SA selection at a particular locus, the products of gene duplication at that particular locus and the following divergence of paralogous genes between the sexes may be positively selected (Connallon & Clark, 2011). The likelihood of gene duplication at SA loci and the rate at which duplicated genes may spread in a population is conditioned both by the strength of SA selection on the ancestral gene, and the patterns of dominance between alleles at this locus. Alternative splicing of the gene products under SA may also evolve as a way to escape SA (Connallon & Clark, 2011). Paralogous genes positively selected under SA selection are expected to accumulate either on the X chromosome, when beneficial in females, or on autosomes when beneficial to males (Connallon & Clark, 2011). The differential expression of SA genes depending on the sex in which they are expressed may also promote the evolution of sexual dimorphism (Ellegren & Parsch, 2007; Griffin *et al.*, 2013; Parsch & Ellegren, 2013; Hollis *et al.*, 2014). This idea assumes different optimal expression levels of SA genes in the two sexes, and that selection for sex-specific up- or down-regulation might drive the evolution of sex-biased gene expression.

Finally, the resolution of sexual conflicts may operate through the evolution of suppressed recombination in a particular genomic region around the sex-determining locus, so that the sex-

linked genes are never found in the genome of the sex in which it is detrimental. This well-known process of sex-specific gene segregation can lead to the evolution of a non-recombining sex-determining region (SDR), located on the sex chromosomes (Charlesworth, 2013; Wright *et al.*, 2016). This region is thus expected to progressively expand by accumulating SA loci, a pattern that has been observed in the sex chromosomes of mammals (e.g., Lahn & Page, 1999), birds (Wright *et al.*, 2014), or some plants (Bergero *et al.*, 2007, 2013). Sex chromosomes are thus predicted to play an important role in the resolution of sexual conflicts by accumulating paralogous genes or SA genes, and by promoting the differential regulation of SA gene expression. However, empirical data tend to show that the putative role of sex chromosomes in the evolution of sexual dimorphism may have been exaggerated (Meisel *et al.*, 2012; Dean & Mank, 2014), or is not as widespread among animals and plants as previously thought.

Is dioecy an evolutionary dead end?

The evolution of separate sexes in plants has long been considered an ‘evolutionary dead-end’ (Westergaard, 1958; Bull & Charnov, 1985; Heilbuth, 2000). Indeed, the widespread distribution of dioecy among angiosperm families contrasts with its relative rareness within individual clades and the lower species richness of dioecy at the species level. To account for this pattern, it has been hypothesized that dioecious species might be more prone to extinction than hermaphroditic ones (Westergaard, 1958; McArthur *et al.*, 1992; Heilbuth, 2000). This hypothesis is congruent with Baker’s law (Baker, 1955), which states that self-compatible hermaphroditism facilitates colonization and long-range dispersal compared to non-selfing dioecious species, so that the former may persist longer, at least in colonizing species. Nonetheless, other factors, such as frequent reversion from separate sexes to hermaphroditism, might also potentially explain the rarity of dioecy (Barrett, 2013; Käfer *et al.*, 2017).

More recent assessments of diversification rates of dioecious lineages have reached contrasting conclusions about the origin of lower species richness in dioecious clades, re-opening discussion over the reasons for the low frequency of dioecy in angiosperms. This recent shift in focus is the fruit of analysis conducted on updated phylogenic datasets and on improved methods to measure diversification rates by Kafer & Mousset (2014) and Käfer *et al.* (2014). These authors drew attention to failure in previous work by Heilbuth (2000) to take into account the fact that derived dioecious clades must be of more recent origin than their hermaphroditic sister clades, which share the ancestral state, so that equal diversification rates between the two sexual systems do not necessarily produce equal species richness.

When relaxing the assumption that dioecy is irreversible, it may be relevant to consider that frequent transitions to and from dioecy will mean that some clades will have originated from a dioecious common ancestor (Käfer *et al.*, 2014). Some angiosperm families are, for instance, well known to comprise only dioecious species (eg. Salicaceae, Menispermaceae, Westergaard, 1958; Renner, 2014). A reassessment of the ancestral sexual system is thus necessary to properly infer diversification rates from sister-clade comparisons, accounting for possible differences in transition rates between sexual systems. Recent studies integrating these potential biases (Käfer *et al.*, 2014; Sabath *et al.*, 2016) may show that the rarity of dioecy is not necessarily due to the more frequent extinctions of dioecious lineages, but that it might be related to its instability and ability to break down without committing the lineage to ultimate extinction (Renner, 2014; Käfer *et al.*, 2017).

Reversions from dioecy to hermaphroditism may have occurred frequently during plant evolution (Renner & Ricklefs, 1995; Barrett, 2013; Käfer *et al.*, 2014; Renner, 2014). David Lloyd (1972, 1975a,b, 1980b) first described monoecious populations of the genus *Leptinella* (*syn. Cotula*) that were derived from dioecious populations. Species in a number of genera are now known to have undergone a similar breakdown in their evolutionary history (e.g., Liston *et al*., 1990; Obbard *et al*., 2006; Volz & Renner, 2008; Schaefer & Renner, 2010). In a recent review, Käfer *et al.* (2017) suggested that the rarity of dioecy might be the outcome of its transient nature. Selection could favor combined sexes over separate sexes and drive populations away from recently evolved dioecy under particular conditions, either as a result of selection on new mutations (fertility mutations instead of sterility mutations), or from standing genetic variation in sex allocation.

When hermaphrodites benefit from the transmission advantage of selfing (Fisher, 1941), with little deleterious effect from inbreeding depression, they may invade sexually dimorphic populations. As noted above, just as dioecy can evolve as a mechanism to promote outcrossing under conditions where $1 + k > 2(1-s\delta)$, i.e., if $s\delta > 0.5$, so hermaphrodites may spread in a dioecious populations when $s\delta < 0.5$. Reduced inbreeding depression may arise from the purging of genetic load following the depletion of genetic diversity in natural plant populations (Byers & Waller, 1999). Demographic events giving rise to genetic bottlenecks during colonization or long-range dispersal can result in populations with reduced inbreeding depression (Pujol *et al.*, 2009). Indeed, populations distributed at the margins of the distribution range of several dioecious species display various levels of hermaphroditism (Yakimowski & Barrett, 2014). Genomic processes such as whole genome duplications may also reduce the overall level of inbreeding depression by maintaining polymorphism between the two non-recombining sub-genomes (Soltis & Soltis, 2000). It is thus

perhaps not surprising that polyploidization appears to be a frequent correlate of sexual-system transitions, both towards and from dioecy (Ashman *et al.*, 2013).

Finally, in situations where mates are scarce, unisexuals might not be able to reproduce at all. This situation can be especially frequent in colonizing species (Baker, 1955), or during long-distance dispersal to a new isolated area, e.g., islands (Baker, 1955, 1967; Pannell *et al.*, 2015). Dioecious species are often found to be 'inconstant' in their sex expression (Korpelainen, 1998), so that females and males occasionally produce pollen or seeds, respectively. Inconstancy in sex expression may be particularly advantageous in situations favouring uniparental reproduction (Baker, 1967), potentially facilitating the colonization of islands for instance. Islands are famously known to be enriched in dioecious species (Carlquist, 1966; Webb *et al.*, 1999) that may have been able to invade these habitats thanks to inconstancy (Pannell *et al*., 2015). Post-establishment selection against high inbreeding depression in these colonizer with uniparental reproduction may have led to dioecy being quickly restored in islands, explaining the currently observed patterns of high frequency of dioecy in these areas (Pannell *et al.*, 2015). Inconstant sex expression is expected to vary depending on the evolutionary pathway a species has followed towards dioecy. In the gynodioecy pathway, the spread of sterility mutations canalizes the development of females, which are thus less likely to deviate from their primary sex function than are males. Such sterility loci directly determine sex at the genetic level (Charlesworth & Charlesworth, 1978a; Charlesworth, 2013). In contrast, dioecious species that have evolved via monoecy may not carry mutations that fully sterilize one function or the other, such that both sexes show imperfect sex expression. It is not rare to observe polymorphism in sex allocation in dioecious populations, underlined by genetic variation (Delph & Lloyd, 1991; Freeman *et al.*, 1997; Korpelainen, 1998). Such inconstant sex expression would provide a strong advantage for isolated individuals, by assuring a minimum of reproduction, so that increased allocation to the opposite sex might sometimes be favored for reproductive assurance. This process has been proposed to explain sexual-system transitions towards gender monomorphism in *Iphigenia novae-zelandiae,* an endemic plant in New-Zealand, which dispersed and evolved from the Australian gender-dimorphic ancestor *Wurmbea biglandulosa* (Case *et al.*, 2008). Gender inconstancy is the most likely precursor to the breakdown of dioecy (Käfer *et al.*, 2017) and has recently received the attention of several theoretical studies (Ehlers & Bataillon, 2007; Crossman & Charlesworth, 2014). In the meantime, evidence for reversions from dioecy to hermaphroditism have accumulated, strongly countering the evolutionary dead-end hypothesis.

Pathways from dioecy to hermaphroditism

Given the important role that gynodioecy has played in the evolution of dioecy (Dufay *et al.*, 2014), it is not surprising that males show inconstant sex expression much more frequently than do females (Ehlers & Bataillon, 2007). As mentioned above, leaky sex expression is typically the outcome of selection for sexual specialization, where sex expression tends to maintain partial sensitivity to environmental cues (Golenberg & West, 2013). The occurrence of inconstancy in both sexes is probably characteristic of species that have evolved dioecy through the monomorphic pathway (Charlesworth & Charlesworth, 1978b; Lloyd, 1980a). Alternatively, inconstancy appears to occur predominantly in males in populations that have evolved dioecy via the gynodioecy pathway, in which females are strictly genetically determined by a male-sterility mutation, so that male inconstancy appears predominant across the angiosperm phylogeny (Ehlers & Bataillon, 2007).

So far, theoretical studies on reversions of dioecy to hermaphroditism have been based on the assumption that it is the males that acquire a female function, and that these modified males potentially spread as hermaphroditic phenotypes (Ehlers & Bataillon, 2007; Crossman & Charlesworth, 2014). For example, Crossman & Charlesworth (2014) derived conditions for the spread of males with a female function in a dioecious population, following previous models that focus on the selfing rate, the level of inbreeding depression, and the compensation factor *k.* Selfing allows the spread of inconstant males when $s\delta < 0.5$, as described in the previous section, resulting in a gynodioecious population (Crossman & Charlesworth, 2014). It is worth mentioning that reversion from canalized sexes to hermaphroditism has long been described in the case of the spread of nuclear restorer genes than can disrupt the action of cytoplasmic male-sterility mutations (Charlesworth & Ganders, 1979; Gouyon *et al.*, 1991). The fitness effects of these restorers, the number of genes involved in fertility restoration, and their molecular mechanism responsible should all affect their mode of transmission and the conditions for their spread in a gynodioecious population, as well as the frequency of females maintained in the population (Charlesworth & Ganders, 1979; Schnable & Wise, 1998).

As noted above whereas gynodioecy can evolve from both hermaphroditism and dioecy, androdioecy is difficult to evolve directly from hermaphroditism, but it may be more likely to originate from the breakdown of dioecy (Pannell, 2002; Wolf & Takebayashi, 2004; Delph & Wolf, 2005; Delph, 2009). This can occur if inconstant females replace canalized females under conditions of pollen limitation, because their occasional production of pollen allows them to self-fertilize (Wolf & Takebayashi, 2004). The pollen function of the inconstant females may increase under selection, but males may nevertheless be maintained with the resulting hermaphrodites under

certain conditions, notably when selection favors hermaphrodites when mates are scarce and males when competition for siring seeds is more intense (i.e., in situations of fluctuating densities; Pannell, 1997). Such situations can be met in a metapopulation context, e.g., in colonizing species that experience high extinction-colonization rates between patches, frequent isolation, and density-dependent selfing rates. Here, isolated inconstant females (or the derived hermaphrodites) may establish a fully hermaphroditic population in which individuals benefit from reproductive assurance conferred by their inconstant phenotype. The resulting monomorphic population may in turn be invaded by specialized males from the original dioecious population, or from other androdioecious populations. Such a process has been proposed to explain the evolutionary maintenance of androdioecy in both ruderal plants and colonizing animals (reviewed in Pannell, 2002).

Overview of the thesis

The above overview has highlighted the fact that transitions between sexual systems involving the breakdown of dioecy are still poorly studied and understood (Käfer *et al.*, 2017). This thesis represents an attempt to understand the selection and evolution of combined versus separate sexes in plants in which monoecy has been an intermediate step in the transition, using the plant *Mercurialis annua* as a model system. It addresses both the evolutionary significance of labile sex expression in the stability of dioecy (Chapters 3 and 4), as well as the implications of sexual specialization in the patterns of dimorphism and sexual conflicts between males and females during sexual-system transitions (Chapters 2 and 5).

In Chapter 2, I characterize the sexes and sexual dimorphism in gene expression in *M. annua,* as well as identifying the timing of divergence between the gene expression profiles of males and females. Sexual conflicts in dioecious plants may be resolved by the differential regulation of gene expression between sexes, and may act as a stabilizer of dioecy, preventing its breakdown. I aim at describing patterns of sex-biased gene expression in a species in which sexes have evolved from a monoecious ancestor via the monomorphic pathway. In particular, I ask whether sex-biased gene expression may influence differences in development between the sexes, in two vegetative tissues, prior to sexual maturity and whether the potential somatic costs of reproduction modify sex-biased gene expression patterns at sexual maturity.

In Chapter 3, I propose a quantitative assessment of gender inconstancy in *M. annua* by placing individuals on the same continuum between pure males and pure females as that proposed by Charnov *et al*. (1976) and Lloyd (1980b). Patterns of labile sex expression and the extent to which it

differs between genders relates to the evolutionary history of dioecy in several ways. First, it is likely to reflect the evolutionary pathway through which dioecy has emerged. Second, sexual specialization and disruptive selection between the sexes, which depend on the strength of sexually antagonistic selection, might condition the patterns of canalization of gender we observe in natural dioecious populations. I undertake between-sex comparisons of inconstancy in a standardized manner, taking into account sexual dimorphism, and discussing them in relation to the potential for labile sex expression be act as a first step in the breakdown of dioecy, via either male or female inconstancy.

In Chapter 4, I describe a natural selection experiment that aimed at manipulating the sex-ratio of experimental lines. Female inconstancy was strongly favoured in the conditions imposed, reflecting the theoretical path in which androdioecy may evolve from dioecy through an intermediate hermaphroditic state (Pannell, 2000). The strength of selective pressures imposed on selected versus non-selected lines resulted in a dramatic difference in the phenotypic response between lines. I analyze, at each of the four generations of selection, the changes in sex allocation brought about by natural selection, and discuss this response in the context of transitions between sexual systems and how inconstancy may bring about such evolutionary shifts.

In Chapter 5, I track the changes in sex-biased gene expression in vegetative and reproductive tissues of *M. annua* from selected lines described in Chapter 4. The resolution of existing sexual conflicts in dioecious populations, and the likely emergence of new conflicts within individuals that have responded to selection through modified sex allocation in both sexual functions, may bring about predictable shifts in differential gene expression between sexes. In particular, I investigate whether, in the absence of a Y-chromosome in females, the phenotypic masculinization of inconstant females during experimental evolution resulted in masculinized expression profiles. The shifts of sex-biased gene expression when moving away from dioecy may also inform us about the nature of sexual conflict in the original dioecious populations, and can help us to understand whether the differences in the expression profiles between males and females suggest ongoing intra-locus sexual conflict, or whether it is rather the signature of past conflicts that has been resolved.

Mercurialis annua as a model for the monoecy-pathway and reversions from dioecy

The *Mercurialis* genus (Euphorbiaceae) comprises approximately ten annual or perennial, wind-pollinated species that are mostly distributed around the Mediterranean Basin (Jovanovic & Cvetkovic, 2010). The genus displays a wide array of sexual systems, including dioecy, monoecy and androdioecy (Obbard *et al.*, 2006a). Apart from sexual-system evolution, the annual mercury clade has been investigated in the past in order to elucidate mechanisms of sex determination

(Russell & Pannell, 2015), patterns of phenotypic sexual dimorphism (Harris & Pannell, 2008; Sánchez-Vilas & Pannell, 2011a,b, Hesse & Pannell, 2011a,b), the effects of environment and hormones on sex expression (Durand, 1963; Louis & Durand, 1978; Hamdi, 1987), the evolution of androdioecy (Pannell, 1997b, 2000; Obbard *et al.*, 2006b), and the relationship between whole-genome duplication and shifts in sexual systems (Obbard *et al*., 2006a; Figure 3c).

Dioecy seems to predate the diversification of the *Mercurialis* genus and is likely the ancestral sexual system in this clade (Obbard *et al.*, 2006a). Evolutionary transitions from separate to combined sexes have been recorded in *M. leiocarpa* (a species mainly located in China) and in the annual mercury clade (Durand & Durand, 1991; Pannell *et al*., 2008; Figure 3c). Sexual-system transitions in this group involve transitions from ancestral dioecy towards monoecy and androdioecy. Confounding factors associated with shifts in sexual system, namely differential geographical distribution (potentially a cause for local adaptation, González-Martínez *et al.*, in review), the demographic history of colonization (Pujol *et al.*, 2009), and whole-genome duplication (Obbard *et al.*, 2006a), often make it difficult to study potential factors influencing sexual-system transitions; the remarkable variation in sexual systems within the *M. annua* species complex allows us to tease apart some of these confounding factors.

In the dioecious lineage of *M. annua*, sex is determined genetically by an XY sex-chromosome system (Russell & Pannell, 2015). Karyotypes of *M. annua* analyzed by Durand (1963) suggest that its sex chromosomes are homomorphic, potentially hinting at a recent origin of dioecy. However, dioecious *M. annua* has long been known to be variable in its sex expression (Vries, 1901; Yampolsky, 1920; Kuhn, 1939), and ‘intergradation’ of the morphs of female flowers were described in detail early on by Yampolsky (Yampolsky, 1920; Figure 3b). Both males and females typically show sex inconstancy, which points to the likely evolution of dioecy via the monomorphic pathway. Sexual dimorphism in several secondary sexual traits has been identified in sexually mature individuals of *M. annua* (Harris & Pannell, 2008; Sánchez-Vilas & Pannell, 2011a,b), underlying the likely presence of sexual conflicts in these lineages. On the other hand, little is known about the sexual conflicts that might exist in monoecious lineages, not least because of the polyploid nature of their genomes.

Early work on sex determination in *M. annua* has demonstrated the specific role of hormone balance in gender development, with auxins and cytokinins having masculinizing and feminizing effects, respectively (Durand & Durand, 1991b). Sex allocation is thus a quantitative trait, most likely influenced by the regulation of hormone balance in *M. annua* individuals. This likely

involves some sensitivity of sex allocation to environmental cues, which may have different consequences depending on gender (Sánchez-Vilas & Pannell, 2014). Previous studies have demonstrated genetic variation for sex allocation in monoecious populations of *M. annua*, showing that this polymorphism may respond to selection in response to density modification by shifting sex allocation towards increased maleness (Dorken & Pannell, 2009). Although true for polyploid monoecious individuals, the genetic variation underlying labile sex expression in dioecious populations of *M. annua* has never been investigated, so that very little is known about the potential for selection on labile sex expression to be a precursor in the transition from dioecy to hermaphroditism, and possibly to androdioecy as well.

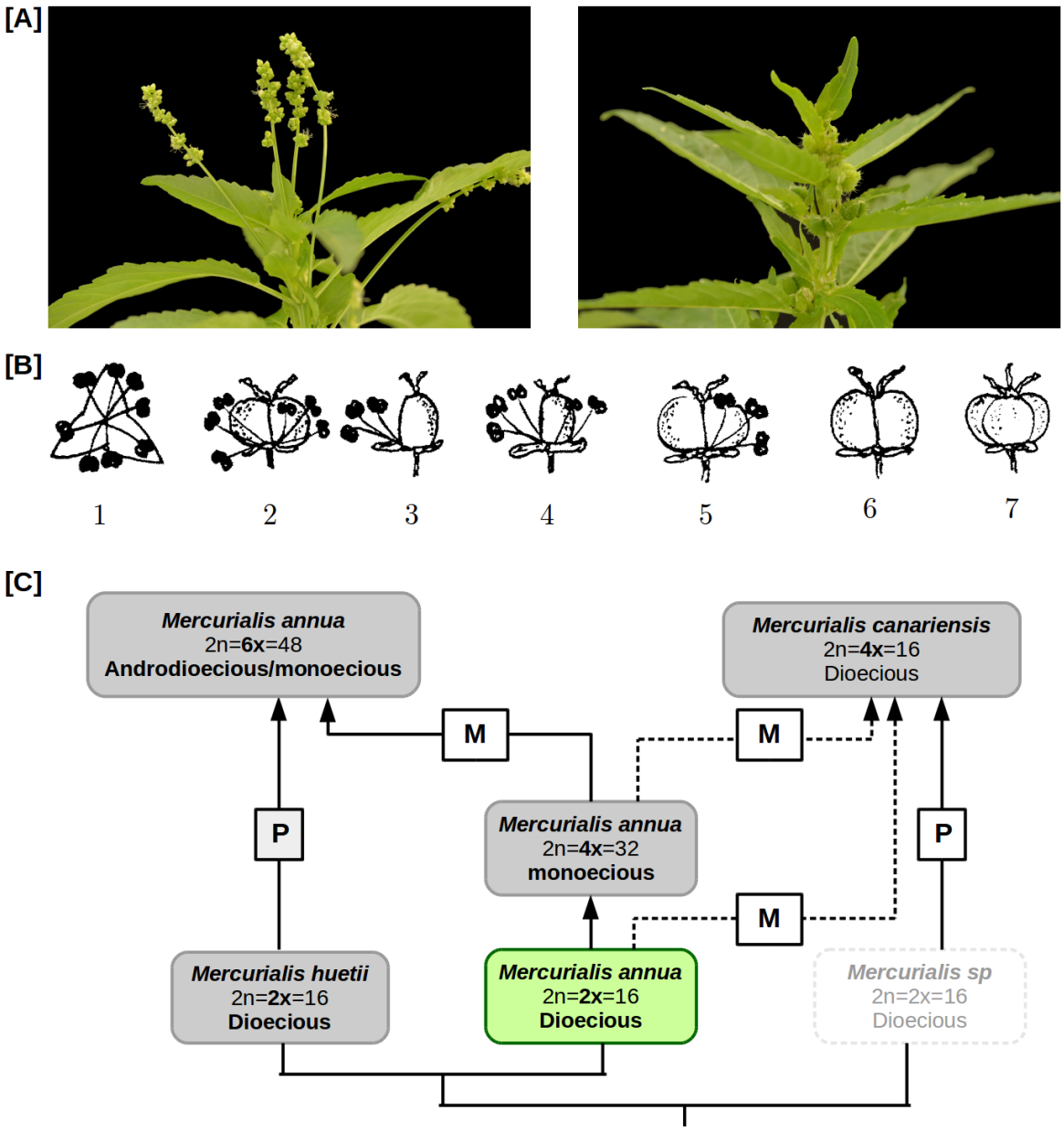

Figure 3. Description of the model species *Mercurialis annua*. **(A)** Photographs of typical male (left) and female (right) inflorescences of *Mercurialis annua*. **(B)** Intergradation of flower morphs in dioecious *M. annua* (taken from Yampolsylsky, 1919). From left to right it features schemes of: 1-male flower, 2-5 - fruits with unusual production of stamen, 6 - two-seeded fruit, 7 - three-seeded fruit. **(C)** Inferred phylogeny of the *Mercurialis annua species* complex (modified from Obbard *et al.*, 2006a). Dashed-lines represent hypothetical relationships. *M* and *P* indicate paternal or maternal origin of polyploid lineages. The dioecious diploid lineage on which this thesis is focused is highlighted in green.

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- CHAPTER 2 -

Sexual dimorphism and rapid turnover in gene expression in pre-reproductive seedlings of a dioecious herb

Guillaume Cossard and John R. Pannell

Introduction

Most flowering plants are hermaphrodites, but separate sexes have evolved frequently in different lineages and are found in about half of all angiosperm families (Charlesworth 2002; Renner 2014). Once partially or fully separate sexes have evolved, males and females may diverge in their phenotypes in response to selection to optimise fitness through each respective sexual function (Bateman 1948; Arnold 1994; Delph *et al.*, 2005; Bonduriansky and Chenoweth 2009; Moore and Pannell 2011; Delph and Herlihy 2012). This process of gender specialisation gives rise to secondary sexual dimorphism. Although sexual dimorphism in plants tends not to be as extreme as it often is in animals (Lloyd and Webb 1977), males and females of dioecious plants almost invariably show some degree of phenotypic divergence in their morphology, phenology, physiology and/or other aspects of their life history (Delph and Wolf 2005; Moore and Pannell 2011; Barrett and Hough 2012).

The differences in the patterns of resource allocation between males and females probably stem primarily from differences between dispersing pollen and maturing and dispersing seeds and fruits. If growth and reproduction compete for resources within an individual, different trade-offs will be set in males and females, which may eventually translate into sexual dimorphism (Reznick 1985; Obeso 2002; Gehring and Delph 2006). This ‘somatic cost of reproduction’ is likely one reason for the smaller size of females at maturity in woody perennial species (reviewed in Barrett & Hough, 2012). In wind-pollinated herbs, by contrast, males are often the smaller sex, presumably as a result of high somatic costs of pollen production (Harris and Pannell 2008). Males and females may also differ in morphology and size as a result of differences in the strength and direction of sexual selection. According to Bateman’s principle (Bateman 1948), female reproduction will be more limited by resources (e.g., for filling seeds and fruits), whereas male reproduction is more likely to be limited by the availability of receptive mates. As a consequence, females may be shaped by selection to optimise the acquisition and deployment of resources during seed and fruit production, while males will evolve strategies that optimise siring success, such as investment in large floral displays and reduced investment in growth and vegetative maintenance (Bond and Maze 1999; Harris and Pannell 2008).

If sexual dimorphism is a consequence of different somatic costs of reproduction, or an outcome of differences in sexual selection, we might not expect to see it expressed much before individuals reach reproductive maturity, i.e., in the earliest stages of plant development, when plants are still seedlings. In plants, as in many other organisms, most mortality occurs at the seed and seedling

stages. At these early stages, males and females undergo similar selective pressure for survival, and deviations from an optimally competitive pattern of allocation are likely to be particularly strongly selected against. Accordingly, males and females should adopt the same strategies of growth. Nevertheless, although we should expect sexual dimorphism to be expressed chiefly in reproductive individuals, there have been several reports of sexual dimorphism for plants in their pre-reproductive, solely vegetative, phase of growth. For instance, males germinate earlier in *Rumex nivalis* (Stehlik and Barrett 2005) and *Silene latifolia* (Doust *et al.*, 1987), and female seeds of *Silene latifolia* tend to enter into dormancy more easily and to experience less mortality when buried (Purrington and Schmitt 1995). Different levels of mortality and competitive ability have been observed in the dioecious grass *Distichlis spicata* (Eppley 2001; Eppley 2005). Although some of these differences might be due to deleterious mutations carried on sex chromosomes (e.g., Smith 1963; Lloyd 1974; Lardon *et al.*, 1999; Stehlik and Barrett 2005), it is also plausible that they reflect adaptive divergence between sexes in anticipation of divergent needs when flowering and fruiting commences.

Whenever it is expressed, secondary sexual dimorphism in species with separate sexes ultimately requires the differential expression of the genome in males and females. In general, this may be brought about in two ways: through sequence divergence of alleles at loci physically linked to the sex-determining locus (Charlesworth 2002; Wright *et al.*, 2016); or through the differential expression of the same alleles in males and females (Ellegren and Parsch 2007; Mank and Ellegren 2009; Griffin *et al.*, 2013; Parsch and Ellegren 2013; Grath and Parsch 2016; Mank 2017). The former implies the evolution of a sex-determination region (SDR) located on sex chromosomes. In plants, the non-recombining SDR is thought to be delimited by complementary sex-determination genes that disrupt the ancestral hermaphroditic sexual system through the expression of linked male and female sterility loci (Charlesworth and Charlesworth 1978). It is widely believed that this region of low recombination should be prone to the accumulation of sexually antagonistic (SA) loci (Fisher 1931; Lewis 1942; Charlesworth and Charlesworth 1978; Rice 1987; Spigler *et al.*, 2008; Charlesworth 2013; Connallon and Clark 2014; Wright *et al.*, 2016). This hypothesis thus requires the sex linkage of at least some genes associated with sexual dimorphism (Zemp *et al.*, 2016).

Ultimately, only a small fraction of genes that show differential expression between the sexes will be in the SDR, because vegetative development in plants is under the control of thousands of genes scattered across the entire genome. Sex-biased expression of shared genes can potentially affect any gene, regardless of its genomic location (Ellegren and Parsch 2007). Such sex-biased expression is thought to be caused mainly by cis-regulatory elements regulated differentially in each of the two

sexes (Williams and Carroll 2009), and which may or may not be related to the sex-determination pathway. Genes involved in sexual dimorphism are thus likely rarely located near the SDR, and the probability of being transferred to it through genome restructuring (duplications, translocations, etc.) must be small. Nevertheless, we do expect sex-linked genes, on average, to play a greater role in sexual dimorphism than genes elsewhere in the genome, and there is increasing evidence that this is indeed the case. For example, the X-chromosome harbour a significant excess of female-biased genes in *Mus musculus* and *Drosophila melanogaster* (Meisel *et al.*, 2012) as well as in *Silene latiolia* in which a subtle feminization and demasculinization of the X-chromosome has been recently highlighted (Zemp *et al.*, 2016).

The differences in gene expression between males and females in vegetative tissues have been investigated in three dioecious angiosperm species: *Silene latifolia* (Zluvova *et al.*, 2010; Zemp *et al.*, 2016), a species with heteromorphic sex-chromosomes (Marais *et al.*, 2008; Delph *et al.*, 2010); *Asparagus officinalis* (Harkess *et al.*, 2015), a species with homomorphic sex-chromosomes (Deng *et al.*, 2012); and *Populus tremula* (Robinson *et al.*, 2014), a species with homomorphic sex chromosome and a reduced SDR (Yin *et al.*, 2008). In *P. tremula* and *A. officinalis*, sex-biased gene expression has been investigated in sexually mature individuals only, when somatic costs of reproduction are fully expressed. In *P. tremula*, Robinson *et al.*, (2014) detected no sexual dimorphism in vegetative traits and found sex-biased expression for only two genes, one of which is located in the SDR and is completely absent in females. In *A. officinalis*, by contrast, 570 genes (i.e., about 0.47% of all loci identified) were shown to be sex-biased, with a large majority of male-biased genes. In *S. latifolia*, the transcriptional patterns of rosette leaves revealed a low degree of sex-biased expression (0.6 % and 0.3% of autosomal genes being female-bias and male-bias respectively). Interestingly, sex chromosomes in this species seem to harbour a greater number of sex-biased genes (4.1 % and 3.4 % of expressed genes are female-biased and male-biased, respectively). The authors found that sex-biased gene expression was overall lower in vegetative leaf tissues compared to flower buds (Zemp *et al.*, 2016).

Sex-biased expression is likely to affect tissues in different ways, by involving different genes or the same genes differently, depending on the specific function of the tissues involved in plant growth. Harris and Pannell (2008) found that males and females of the plant *Mercurialis annua* differ in terms of their relative allocation of biomass to roots versus shoots. In particular, males, in which pollen production is thought to be mainly limited by nitrogen availability, tend to invest more heavily into roots than do females, and males are consequently typically the smaller sex above ground (Harris and Pannell 2008; Sánchez-Vilas and Pannell 2011a; Labouche and Pannell 2016; J.

Tonnabel J, David P and Pannell JR, unpublished data). Roots and shoots are known to have very different profiles of gene expression in *Arabidopsis thaliana* (Schmid *et al.*, 2005), but the extent to which such tissue-specific differences in morphology or allocation are reflected by tissue-specific gene expression is, to our knowledge, so far not known.

Patterns of sex-biased gene expression are also likely to vary with the stage of development at which it is measured. Schmid *et al.* (2005) investigated expression profiles during seed development in *Arabidopsis thaliana* and observed “strong modulation of gene expression along the time axis”. In species with separate sexes, therefore, we might expect expression profiles to be highly dynamic, too, with potentially different dynamic patterns shown between the sexes. Although it is clear from the papers cited above that some dioecious species have evolved substantial sex-biased gene expression, we have a poor idea of when these difference might begin during ontogeny, or (and in particular) how the differences might change over time. In the only study to have addressed this issue (to the best of our knowledge), Lipinska *et al.* (2015) found that sex-biased gene expression differed before and after sexual maturity in the haploid dioecious brown alga *Ectocarpus siliculosus*. Surprisingly, they found that more genes were sex-biased before (4.62 % and 8.22% for female-biased and male-biased genes, respectively) than at sexual maturity (1.23 % and 2.25 % for female-biased and male-biased genes, respectively) (Lipinska *et al.*, 2015). The generality of such a pattern is unknown.

Genes that are involved in sex-biased gene expression are thought to evolve faster than non-biased genes. This could be due to stronger sex-specific positive selection, or to relaxed constraints imposed on these genes (Ellegren and Parsch 2007). Because intra-sexual competition in males is probably stronger than it is in females, male-biased genes are expected to be under stronger sexually antagonistic selection than female-biased genes, and to show higher rates of protein-coding evolution (Ellegren and Parsch 2007). Differences in the rate of evolution between male-biased and female-biased genes have been found in the animal *Drosophila melanogaster*, with particularly rapid evolution of genes involved in spermatogenesis in males (Haerty *et al.*, 2007), but female-biased genes have been found to evolve faster in *D. pseudoobscura* (Assis *et al.*, 2012). The relationship between sex bias and evolutionary rates of nucleotide change in protein-coding genes remains even more poorly known in flowering plants. In *Silene latifolia*, male-biased genes do not seem to evolve faster than female-biased ones (Zemp *et al.*, 2016), possibly because male bias is primarily the result of a down-regulation of the affected genes in females rather than a change in expression level in males (Zemp *et al.*, 2016). Similarly, in the brown alga *E. siliculosus*, Lipinska

et al., (2015) found that male- and female-biased genes appeared to be evolving at the same rate under positive selection.

Here, we present an analysis of patterns of gene expression in the dioecious wind-pollinated herb, *Mercurialis annua*, over the course of its early growth, from soon after germination to the time of flowering, and in both its shoots and roots. We (1) document patterns of sex-biased gene expression in its above- and below-ground tissues, and (2) ask in particular whether they are apparent before sexual maturity is reached. We also (3) test the hypothesis that sex-biased genes are found more commonly on the sex chromosomes than expected by chance, and (4) ask whether sex-biased genes in general evolve faster than non-biased ones. Although *M. annua* shows striking variation in its sexual systems (Pannell *et al.,* 2008), dioecy is ancestral in the genus and species (Obbard *et al.*, 2006), and all species of the genus with separate sexes show sexual dimorphism in a number of traits. These traits include: a higher germination rate of seeds bearing a male embryo (Gillot 1924a in Lloyd and Webb, 1977); earlier flowering in males (Harris and Pannell 2008); males with a smaller size but a greater height/biomass ratio (Hesse and Pannell 2011) and a greater root/shoot ratio than females (Harris and Pannell 2008); greater vulnerability of males to herbivores (Sánchez-Vilas and Pannell 2011a); differences between the sexes in within- and between-species competitive abilities (Sánchez-Vilas *et al.*, 2011; Orlofsky *et al.,* 2016); and large differences between the sexes in the deployment of resources such as carbon and nitrogen (Sánchez-Vilas and Pannell 2011b). The genome and transcriptome of dioecious *M. annua* have recently been assembled and annotated, with the identification of the sex chromosome and the discovery of several hallmarks of Y-chromosome degeneration (Ridout K *et al.,* unpublished data).

Materials and methods

Study species

Mercurialis annua is a wind-pollinated annual herb in the Euphorbiaceae family, with a broad distribution across Europe (Durand 1963; Durand and Durand 1992; Pannell *et al.,* 2004). Populations in most of its range are diploid, exclusively dioecious and exhibit a 1:1 sex ratio (Yampolsky 1919; Russell and Pannell 2015). Males and females differ in a number of reproductive and vegetative traits. Males bear their flowers on erected stalks (peduncles), while females produce axillary flowers (Pannell 1997a; Pannell 1997b; Pannell *et al.*, 2008). Males tend to flower earlier than females, to invest more heavily in reproduction, and to have a higher root/shoot ratio than females (Harris and Pannell 2008). At the seed stage, a slight difference in germination time between males and females has been observed by Gillot (1924a, in Lloyd and Webb, 1977). As observed in most sexually dimorphic short-lived herbs species (Barrett and Hough 2012), males of

M. annua are smaller than females. This is likely due to their greater resource allocation to roots and to the investment of nitrogen to copious pollen production at the expense of shoot growth. The marked sexual size dimorphism observed in the species is thus probably largely attributable to diverging costs of reproduction between males and female (Harris and Pannell 2008).

Study material

For our study, we used the F1 progeny of an experimental population of seed collected from 25 populations across the dioecious range of *M. annua* in western and eastern Europe, which we thoroughly mixed and sowed together in seed trays. At early flowering stage, we randomly chose males and females and assigned them to three plots outside, each composed of 90 males and 90 females. The F1 seeds from females of all three populations were harvested for the experiment after seven weeks of growth.

The plants were all germinated and grown in a growth chamber under constant temperature, moisture and light conditions. For Experiment 1, plants were grown in soil with slow release fertilizer (at 4 g per litter of soil). We harvested plants for RNA extraction at four developmental stages, each corresponding to the growth of a new pair of leaves, with stage I representing seedlings with cotyledons and the first pair of true leaves. Stages I, II, and III comprised individuals that had not yet begun to produce floral buds. We then waited two full weeks before sample fully flowering individuals at stage IV. We froze tissues immediately in liquid nitrogen. Tissues were then stored at -80°C until RNA extraction. As individuals cannot be phenotypically sexed before flowering, extra material was collected from each plant for genotyping and sex determination (see below). RNA was extracted from pools of 10 individuals (see below), with 3 pool replicates per sex and per stage considered.

For Experiment 2, two-week old germinated seedlings were transplanted into pots filled with Seramis clay granules for further growth under hydroponic conditions (allowing roots to be sampled without extensive washing, thus increasing considerably the quality of RNA extracted). Roots and leaves of five individuals of each sex were separately sampled at stages III and IV, defined as for Experiment 1.

DNA extraction and SCAR amplification to determine individual sex

We determined the sex of each individual sampled for RNA extraction on the basis of the presence or absence of a Y-linked genetic ‘SCAR’ (sequence-characterised amplified restriction) marker (Khadka *et al.,* 2002). Total genomic DNA was extracted from fresh or silicagel-dried leaves

according to the protocols of the DNeasy Plant Mini kit (Qiagen) or of the NucleoSpin® PlantII kit (Macherey-Nagel). Following Russell and Pannell (2015), we checked for DNA integrity through the amplification of a neutral 766bp marker. Plant sex was then determined by the amplification of OPB01-1562 (Khadka *et al.*, 2002). PCRs were performed in a volume of 20 µL containing 10 µM of each dNTP, 50 µM of MgCl2, 20 µM of each primer and 1 unit of TaQ DNA polymerase (Eurobio laboratories)

mRNA extraction and sequencing

We extracted total RNA from each sample using RNeasy miny kit (Qiagen), following the manufacturer recommendations. The quality of extracted RNA was determined using a fragment analyser, and concentrations were measured using Qubit (at the Center for Integrative Genomics, 'CIG', University of Lausanne). We prepared RNAseq libraries using TruSeq Stranded mRNA Sample Prep Kit (Illumina), following the manufacturer's protocol, along with SuperScriptII (Invitrogen) enzyme for retrotranscription, as advised by Illumina. Library quality was checked using BioAnalyser at the CIG (Lausanne) before being sequenced on an Illumina Hiseq 2000 as paired-end 100 bp reads.

Genome annotation

For genome annotation, we used the assembled *Mercurialis annua* genome sequence version 1.4 (Ridout K *et al.*, unpublished data), masked for transposable elements and repeat tandem libraries from *Mercurialis*, *Euphorbiacae*, *Vitis vinifera* and from the Plant Genome and System Biology repeats database *(http://pgsb.helmholtz-muenchen.de/plant/recat/)*. Reads were mapped onto the assembled *M. annua* genome (Ridout K *et al.*, unpublished data) with Tophat v2.0.13 (Trapnell *et al.*, 2012), which uses bowtie2 to align reads and infer splice-junctions. Multi-mapping was allowed, and only concordant mapping was reported. The resulting transcripts were then assembled for each sample using cufflinks 2.2.1 (Trapnell *et al.*, 2012), correcting for reads that mapped at different locations in the genome. The transcriptome assembly was produced from extensive RNAseq data (including transcripts of all libraries analyzed in the two experiments of the present study, for a total of 1.63 billions pairs of 100bp reads) using Cuffmerge with default parameters (Trapnell *et al.*, 2012). These assemblies were used as input for the gene predictor software Augustus 3.0.1 (Stanke *et al.*, 2006), with parameters trained for *M. annua* (from Ridout K *et al.*, unpublished data). Gene prediction resulted in 34,006 genes with correct start codon and frame. RNAseq data allowed us to identify a number of alternative isoforms for some of these genes, resulting in 37601 transcripts in total. These reads were all kept as a reference transcript dataset for downstream analysis of differential expression (Brown *et al.*, 2016).

Differential expression analysis

Data were assessed for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters were clipped from the resulting sequences with Trimmomatic (Bolger *et al.*, 2014), which we also used for quality filtering: reads were trimmed if the leading or trailing base had a Phred score < 3, or if a sliding window average Phred score over four bases was < 15. We then discarded all unpaired reads and all pairs of reads in which at least one pair was < 36 bases long, leaving on average more than 25 million paired-end reads per sample.

Transcript abundances were estimated with Kallisto (Bray *et al.*, 2015) using 31 bp-long kmers to pseudo-align all trimmed reads onto the annotated gene set (including all transcripts) produced for *M. annua*. As described in Soneson *et al.*, (2016), transcript abundances were then summed up within genes and multiplied with the total library size in millions, using the tximport package (Love *et al.*, 2016). This resulted in scaledTPM (scaled transcript per million), which we refer to in all figures and tables, and count estimates per gene. Count matrices were used as input to estimate differential expression using DESeq2 package (Love *et al.*, 2014). An adjusted p-value cut-off of 0.05 was applied for all analysis.

Functional annotation of differentially expressed genes

We performed a BLAST search (tblastx 2.2.29+) of all mapped transcripts against a custom protein database of all eudicots (E-value cutoff of 1e-05, 20 hits retained). Functional categories were attributed by mapping GO terms using Blast2GO 3.0 (Conesa *et al.*, 2005). We also performed an identification of conserved protein domains among these putative genes using InterProScan (Quevillon *et al.*, 2005). Finally, in order to infer any evidence for sex-specific or stage-specific enrichment in particular GO-terms, we performed Fisher exact tests, implemented within B2GO (FDR < 5%, Benjamini and Holchstein multiple-testing correction; Conesa *et al.*, 2005).

Measure of evolutionary rates and detection of selection

We estimated evolutionary rates by comparing *M. annua* sequences with orthologs from its sister species *M. huetii*, for which we grew a male and a female in the greenhouse of the University of Lausanne in summer 2015. We sampled seven different tissues from each of the *M. huetii* individuals (cotyledons, leaf before and after flowering, stem, roots, apical meristem and flowers) and extracted RNA from pooled tissues separately for each individual. Library quality was checked using BioAnalyser at the CIG, University of Lausanne, before being sequenced on an Illumina Hiseq 2000 as paired-end 100 bp reads. We assembled a reference transcriptome for *M. huetii* by

combining *de novo* assembly using Trinity (Haas *et al.*, 2013) and a genome-guided assembly using the *M. annua* genome as a reference. Redundancy in the combined transcriptome was assessed using CD-HIT-EST v4.6.1 (Li and Godzik 2006). The reduced assembled *M. huetii* transcriptome contained 31,979 genes. We used reciprocal blast (with an e-value cutoff of 1 $x10^{-6}$) to find orthologous genes between *M. annua* and *M. huetii* transcriptomes. Using the *PopPhyl* pipeline (Tsagkogeorga *et al.*, 2010; Romiguier *et al.*, 2014), we inferred the median degree of synonymous (*dS*) and non-synonymous divergence (*dN*) between *M. huetii* and *M. annua* from the raw reads of the 20 individuals sequenced separately in Experiment 2, for each ortholog. We tested the difference in the *dN/dS* ratio between female-biased, male-biased and non-biased genes using ranked Mann-Whitney tests.

Sex linkage of genes involved in sex-biased expression

To attribute possible sex linkage for the genes in our analysis, we used a previously established database (Ridout K *et al.*, unpublished data) of 527 putative sex-linked contigs in *M. annua*, using SEX-DETector (Muyle et al, 2016). We thus mapped our genes against this database using reciprocal BLAST and reported all results with more than 90% similarity (using an e-value cut-off of 1 $x10^{-6}$).

Results

To investigate sex-biased gene expression in *Mercurialis annua*, we conducted two related experiments. The first addressed sex-biased gene expression in apical tissue at four different developmental stages. The second focused on two key developmental stages investigated in two vegetative tissues: roots and mature leaves. All our analyses and estimated transcript abundances are based on a reference transcript dataset of 34,006 protein-coding genes produced from an EST-guided gene annotation pipeline run on the assembled genome of *M. annua* version 1.4 (Ridout K *et al.*, unpublished data), taking into account eventual alternative splicing (Brown *et al.*, 2016).

Experiment 1: Stage-dependent patterns of sex-biased gene expression

Sex-biased gene expression

On average, across all stages of development, we found 27,978 genes that were expressed in apical tissues of *M. annua* (Table S1). A total of 231 genes were expressed differently between males and females for at least one of the four stages of development assayed (i.e., ~ 0.83% of expressed genes). For each stage considered, there were more male-biased than female-biased genes (Figure 1). Sex-specific expression concerned only a minority of the genes expressed, at most 0.14 % and

0.12 % for male and female-specific genes, respectively (Table S1). The first two stages of growth showed the fewest differentially expressed genes (DEG), with a total of 12 genes at stage I, and 13 genes at stage II. The greatest number of DEG was observed at stage III (170 genes), just before flowering (Figure 1; Table S1). At stage four, after the onset of flowering, 64 genes were sex-biased.

We further explored details of differential gene expression between biased and non-biased genes in males and females for each of the four stages of development (Figure 2). Sex-biased genes were significantly down-regulated in females at stage II. Interestingly, males and females showed an increase in the expression of sex-biased genes just before flowering, which continued in mature individuals. This led to a significant over-expression of sex-biased genes compared to non-biased ones in both sexes at these stages (Figure 2; Table 2). At all stages investigated, we observed that male-biased genes had a greater mean expression than female-biased genes. However, taking into account all expressed genes, the overall pattern of higher expression in males seems to be reverted from stage III on, (Figure S1), coincidently with the increased degree of SBGE. Most of the sex-biased genes showed bias at only one of the stages sampled (Figure 3). No gene continuously displayed a female-bias or male-bias across the four stages sampled.

Functions and identity of sex-biased genes

We conducted gene ontology analysis, using Blast2GO (Conesa *et al.,* 2005), to assess possible enrichment for specific functions of DEG at each developmental stage sampled. No significant enrichment in GO terms was detected in sex-biased gene sets of the two earliest stages of plant growth. At stage III, female biased-genes were principally enriched for functions related to photosynthesis/chloroplastic processes, including starch metabolism (Table 3). Similarly, at the same stage, male-biased genes were enriched for acetyl-coA-related functions, i.e., fatty acid synthesis that occurs in plastids. In sexually mature individuals (stage IV), female-biased genes showed no specific enrichment, while male-biased genes were enriched for a number of functions, mostly related to the regulation of transcription, among which we found the regulation of gene expression (GO:0010468), protein binding (GO:0005515) and floral meristem determinacy (GO:0010582).

We identified two orthologs among the 13 protein-coding transcription factors involved in plant reproduction (described in Table 1 of Pajoro *et al.,* 2014), based on retention of reciprocal hits and with an e-value cut-off of 1×10^{-6}. The two genes were male-biased in our experiment and are thought to be directly involved in plant reproduction and the determination of floral meristems: *LEAFY* (*LFY*) and *APETALA1* (*AP1*). *AP1* was male-biased from stage II to stage IV, while *LFY*

was male-biased at stages III and IV only (Figure 4). Both genes showed similar levels of expression in young plants at stage I and were uniformly up-regulated in both sexes during plant development, to higher levels in males compared to females (Figure 4). Along with these two genes, we also identified the sex-biased expression of an ortholog of *GLOBOSA* (*GLO*), and promoters of *SQUAMOSA* (SQUA), both of which are involved in floral determinacy. The former showed male-biased expression in our experiment (g10857), as did the two promoters of a *SQUAMOSA-like* genes, g1783 and g10599, which were male-biased at stages III and IV, respectively (Figure S2). In addition, g15976, which blasts against *AGAMOUS-like* isoform 6, was always more highly expressed in males than in females, in which its expression began only at stage III. Finally, an *AGAMOUS-like* isoform x4 (g7084), as well as an ortholog of *CRABS CLAW* (*CRC*) gene (g21428), were found to be female-biased at stage IV, and at stages III and IV, respectively (Figure S2).

Experiment 2: Tissue-dependent patterns of sex-biased gene expression

On average, we found of 30,192 genes that were expressed in leaves and roots of *M. annua* (Table S2). Most of the variance in our expression data could be accounted for by differences across tissues and stages (Figure S3). Male and female samples clustered together in a PCA analysis, indicating that differential expression between the sexes was much less conspicuous than between tissues or development stages. The majority (c.a., 89.7 %) of genes expressed were detected in both roots and leaf tissues (Figure S4), so that tissue specificity of transcripts was low. When we compared expression patterns between tissues, we found that a total of 16,038 and 15,234 genes were differentially expressed between tissues before and after flowering, respectively.

In total, we identified 789 DEG in Experiment 2 (about 2.61 % of expressed genes), i.e., substantially more than in Experiment 1. The number of DEG in roots was low and remained relatively unchanged before and after flowering (77 and 63 DEG, respectively). In contrast, patterns of sex-biased gene expression changed more dramatically in mature leaves (Figure 5), with a total of 156 and 597 sex-biased genes before and after flowering, respectively (Table S2). This trend was largely attributable to a drastic increase in the number of female-biased genes in mature leaf tissues, from 48 to 430 genes before and after flowering, respectively (Figure 5). Sex-biased genes were largely sex- and tissue-specific (Figure 6). Male-biased genes in root tissues did not seem to be enriched for any particular function, either before or after flowering (Table 4). Female-biased genes in roots before flowering were involved in stress response to the presence of inorganic compounds such as copper ions, which may be linked to the hydroponic growth of our samples in Experiment 2. At the same stage, leaves showed enrichment for various metabolic processes, including the activity of the phosphoethanolamine N-methyltransferase (GO:0000234), which plays a role in sterility

sensitivity in *Arabidopsis* (Mou *et al.*, 2002). Female-biased genes in leaves of sexually mature individuals were differently enriched for cell-associated processes involving the cytoskeleton. No enrichment could be detected for male-biased genes in leaves of mature individuals.

Evolutionary rates of sex-biased versus non-biased genes

Reciprocal blast comparison of the *M. annua* transcriptome with that of the closely related species *M. huetii* identified 8,675 orthologous genes (~ 28.7 % of genes expressed in Experiment 2). We kept for further analysis genes with non-zero *dN/dS* values and *dS* > 0.0637341 following the PopPhyl pipeline, resulting in 112 female-biased, 46 male-biased and 4,346 non-biased genes (Table 5). We detected no statistically significant difference of *dN/dS* between female-biased, male-biased and unbiased genes, revealing no specific selection acting preferentially on sex-biased compared to non-biased genes.

Putative sex linkage of sex-biased genes

We asked whether the sex-biased loci found in our study were sex-linked, based on an analysis of contigs previously identified in *M. annua* (Ridout K *et al.,* unpublished data). A previous analysis conducted over two generations on two families of diploid *M. annua* identified 527 sex-linked genes that were inherited in a sex-specific manner (Ridout K *et al.,* unpublished data). Among the total of 972 sex-biased genes identified in both experiments, a subset of 35 genes (4.15%) were found among the sex-linked genes, representing 7.02% of sex-linked genes known to date. This supports a preferential linkage of sex-biased genes on sex-chromosomes (Fisher-exact test, $P = 4.51 \times 10^{-4}$)

Discussion

Low proportion of genes with sex-biased gene expression in plants

We detected SBGE in the three vegetative tissues investigated, and in both our experiments. Despite this qualitative uniformity, we found differences between the two experiments in terms of the number of sex-biased genes and their identity. Such variation is not particularly surprising, given that plants from the two experiments differed markedly in terms of the substrates they were on (soil and peat in Experiment 1 versus hydroponic conditions in Experiment 2), and given that plants were grown at different times. Plant development is known to be strongly plastic, especially regarding resource acquisition adjustments in response to nutrient availability (Sultan 2000; Weiner 2004). Typically, the production of shoot tissues is accentuated in resource-rich environments, while root tissues represent a larger part the overall plant biomass in resource-poor environments (Gedroc *et*

al., 1996; Sultan 2000). In *Arabidopsis thaliana,* variation in water availability during growth is known to affect the expression of a number of genes (Seki *et al.,* 2001).

We found that, of the 28,372 genes expressed across all the tissues we sampled, between 0.83% (Experiment 1) and 2.61% (Experiment 2) showed sex-biased expression, representing a total of about 3.33% of expressed genes involved in SBGE. In comparison, the proportion of SBG identified in vegetative tissues of other plant species investigated to date ranges from 0.0065% of expressed genes (only two genes were significantly sex-biased) in *Populus tremula* (Robinson *et al.,* 2014), to about 0.9% of the genes expressed in both sexes in *Silene latifolia* (Zemp *et al.,* 2016). SBGE is typically lower in vegetative than in reproductive tissues of dioecious plants (Zemp *et al.,* 2016). For instance 2.7% and 1.07% of all genes expressed in floral tissue showed SBGE in *Salix suchowensis* (Liu *et al.,* 2013) and *Cucumis sativus* (Guo *et al.,* 2010), respectively.

The low SBGE observed in plants contrasts with that found for other taxonomic groups, particularly animals (Yang *et al.,* 2006; Eads *et al.,* 2007; Mank *et al.,* 2010; Prince *et al.,* 2010; Baker *et al.,* 2011; Hale *et al.,* 2011; Kang *et al.,* 2011; Naurin *et al.,* 2011; Assis *et al.,* 2012; Catalan *et al.,* 2012; Martins *et al.,* 2013; Pointer *et al.,* 2013; Albritton *et al.,* 2014; Jansen *et al.,* 2014; Smith *et al.,* 2014; Stuglik *et al.,* 2014; Wong *et al.,* 2014; Chain 2015; Jiang *et al.,* 2015; Liu *et al.,* 2015; Wang *et al.,* 2015; Chauhan *et al.,* 2016; Huylmans *et al.,* 2016; Machado *et al.,* 2016; Mueller *et al.,* 2016; Poley *et al.,* 2016). One possible reason is that separate sexes in the animals investigated so far are much older than in the plants for which we have data (Charlesworth 2013). Although the time since the evolution of separate sexes could partly explain differences in SBGE among lineages, differences in gene expression between males and females of the plant *S. latifolia* and hermaphrodites of its close relative *S. vulgaris* (Zemp *et al.,* 2016) confirms that SBGE can evolve quickly, so other explanations are probably needed. For instance, plants have a vegetative modular development that is similar between sexes (e.g., Grant *et al.,* 1994), from the polarisation of the root-shoot axis during seed development to the process of branching, whereas animals develop a separate germline early and display deep structural variation between sexes from the earliest stages of development (e.g., Rinn and Snyder 2005). Another reason for the low SBGE in plants relative to animals concerns peculiarities of mating that affect the context of sexual selection. In animals both intra- and inter-sexual selection may contribute to divergence between the sexes whereas the opportunities for inter-sexual selection (female choice) in plants are necessarily more limited and probably confined to the potential choice by pistil tissue among male gametophytes, if at all (Stephenson and Bertin, 1983; Arnold 1994; Skogsmyr and Lankinen 2002; Moore and Pannell 2011).

While the differences between plants and animals in their development and mating may explain some of the differences observed in SBGE, the explanations are not adequate for the differences observed between angiosperms and brown algae, which share with land plants a modular developmental program, a sessile habit, indirect mating behaviour and the lack of a germ line (Lipinska *et al.*, 2015). It is not known when gametophytes of *Ectocarpus* evolved separate sexes, but sex chromosomes started to diverge at least 70 Ma (Ahmed *et al.*, 2014). Interestingly, up to ~12.7% of genes expressed in the brown alga *Ectocarpus siliculosus* are sex-biased, even in sexually immature individuals (Lipinska *et al.*, 2015). Although brown algae share important traits with plants (not least their modular construction and their ability to photosynthesise), they are phylogenetically as close to animals as they are to plants. Separate sexes in *Ectocarpus* occur in the haploid gametophyte phase of the life cycle, not in the diploid phase as in flowering plants. Moreover, both their (U and V) sex chromosomes have a sex-specific non-recombining region (Ahmed *et al.*, 2014), allowing them to diverge more readily from one another than X and Y or Z and W might do (Immler and Otto 2015). *Ectocarpus* genes that showed female-biased expression in mature plants are preferentially located on the sex chromosomes, whereas female-biased genes in premature plants are not. Not only does the proportion of sex-bias thus differ in *Ectocarpus*, but so does their genomic location and the temporal dynamics of their expression.

Sex-biased gene expression in shoots versus roots

We found differences in SBGE between root and leaf tissues, and in the way gene expression changed during development. Leaf tissues in *M. annua* had more SBG than did roots during solely vegetative growth. There was also a burst of SBG when sexual maturity was reached (especially for female-biased genes; Figure 5), in leaves but not in roots. These patterns could be partly due to lower number of cell types in leaves than in roots, but fundamental differences in physiology between roots and shoots likely play a role. Although male and female functions require different provisioning of nitrogen and water that might place divergent demands on the roots of males versus females (Harris and Pannell 2008), it is likely that above-ground growth and reproduction is subject to more strongly differing trade-offs between the sexes than below-ground growth.

Turnover of sex-biased gene identity

Our results reveal a high turnover of genes involved in SBGE during plant development in both roots and shoots of *M. annua*, i.e., the recruitment of different genes, or a differential timing of expression of the same genes, between males and females during growth. We found SBGE as soon as the first whorl of leaves was produced, and might well have been present as early as the seed

stage. During their vegetative stage of growth, plants may benefit by preparing themselves for later sex-specific phenotypes at flowering, both in terms of resource acquisition and allocation traits. Such a strategy might involve differential investment in roots versus shoots (Gedroc *et al.*, 1996), or differential growth rates and timing of flowering. In *Rumex hastatulus*, for example, males are larger than females at the time of flowering, i.e., when pollen needs to be dispersed, but the pattern becomes reversed after fertilization, presumably with advantages for seed dispersal (Pickup and Barrett 2012).

The timing of flowering may influence sexual dimorphism through a differential onset of somatic costs of reproduction in the two sexes. We observed that the number of SBG peaked just before, and after, sexual maturity (Figure 1). Because the somatic cost of reproduction should affect vegetative growth most after a differential investment in reproduction, we expected and observed more SBG in sexually mature individuals (0.23% of expressed genes at stage IV) compared to the first stages of development (0.04% and 0.05 % of expressed genes at stages I and II, respectively). However the number of SBG just prior to flowering (stage III) was even higher (about 0.60% of expressed genes), perhaps reflecting a developmental preparation for the ensuing sex-specific transition to flowering. This might be expected especially for species like *M. annua*, *Spinacia oleracea*, or *Cannabis sativa*, in which rudiments of the opposite sex are absent within flowers and where meristems differentiate differently for male versus female flowers early on (Lebel-Hardenack and Grant, 1997), likely via the differential expression of floral identity genes (e.g., ABC genes; Weigel and Meyerowitz, 1994). In this context, it is perhaps relevant that the genes *LEAFY* and *APETALA1* were differentially expressed between the sexes before flowering occurred (stage IV) in terms of male-biased expression (Figure 4). It is of course possible that the earlier flowering by males of *M. annua* is not directly related to meristem identity determination, yet might still be associated with sex-biased gene expression prior to sex organ differentiation.

Genomic location of sex-biased genes

We found that about 7.02% of the known sex-linked sequences in *M. annua* (Ridout K *et al.*, unpublished data) showed SBGE. This result indicates that sex chromosomes seem to harbour an excess of SBG and thus to play a more important role than autosomes in SBGE, as commonly expected (Rice 1984; Kirkpatrick and Hall 2004; van Doorn and Kirkpatrick 2007; Dean and Mank 2014). For instance the Y chromosome of *Silene latifolia* appears to harbour QTLs responsible for turnover of sex chromosomes induced by sexual conflicts or sexually dimorphic traits such as flower number and photosynthetic rate (Delph *et al.*, 2010), and 4.09% of all sex-biased genes in

flower buds were found linked to the sex-chromosome 19 in the willow *Salix suchowensis* (Liu *et al.*, 2013).

Evolutionary rates of sequence change for sex-biased genes

We found no significant differences in the evolutionary rates of sex-biased and unbiased genes (Table 5). Male- and female-biased genes were also found to have been evolving at about the same rate in *M. annua*, so that biased expression in the vegetative tissues sampled (apex, leaves and roots) is not associated with an accelerated rate of nucleotide substitutions. Such tissues are shared between sexes and express genes that are neither sex-limited nor sex-specific in their expression (Table S1). It has been suggested that the breadth of expression of sex-biased genes should influence the rate of their evolution (Meisel *et al.*, 2012). However, the relationship between the tissue specificity of expressed genes and sex-biased or sex-specific expression is probably quite complex (Dean and Mank 2016), not least because of constraints imposed by pleiotropy on the evolution of genes expressed in different tissues. Indeed, Meisel *et al.*, (2012) found that the overall pattern of accelerated evolution of sex-biased genes in *Drosophila melanogaster* and *Mus musculus* was attributable to genes with tissue-specific expression. In *Ectocarpus siliculosus*, sex-biased genes were found to have a significant tendency to be more narrowly expressed in tissues and developmental stages, and evolutionary rates were higher for those genes (Lipinska *et al.*, 2015). In contrast, most genes in *M. annua* are expressed throughout development, albeit to a varying degree, so that the absence of evolutionary rate heterogeneity as a function of expression patterns is perhaps not surprising.

Concluding remarks

In contrast with many animals, and consistent with the few plant species studied to date, dioecious *M. annua* shows rather limited SBGE, However, it is striking that males and females of *M. annua* begin to express a number of their genes differently at the earliest stages of vegetative development. Most of these genes are on autosomes, not the sex chromosomes, and are likely responsive to upstream signals emanating from a (yet unknown) sex-determining locus that is expressed very early in development. Given that other dioecious plants show life-history differences between males and females at the seed and seedling stage (Barrett and Hough 2012, and see Introduction), it seems likely that the early expression of sex-determination and relevant downstream loci will be a common feature of dioecious plant development.

The evolutionary genetics of sex determination is understood for very few dioecious plants (e.g., (Boualem *et al.*,; Akagi *et al.*, 2014; Akagi *et al.*, 2016), and we remain almost completely ignorant

of how sex is determined in *Mercurialis annua*. Given that both males and females of *M. annua* occasionally produce flowers of the opposite sex (Yampolsky 1920; Gillot 1924b; Cossard G and Pannell JR, unpublished data), it seems unlikely that sex expression is due to the expression of sterility mutations in genes on the sex chromosomes. Rather, sex differentiation is likely governed by one or more genes on the Y chromosome mediated by endogenous hormone signalling. The details of this process in *M. annua* are not understood, but exogenous application of cytokinin causes males to produce pistillate flowers and seeds (Louis and Durand 1978; Hamdi 1987; Eppley and Pannell 2009), and similar compounds may play a role in sex determination and differentiation between males and females at the earliest stages of development. Given that male and female fitness components likely depend on different plant growth and allocation strategies in *M. annua*, it is plausible that the early divergence in gene expression we have observed here reflects optimization of the plant phenotype long before flowering.

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FIGURES

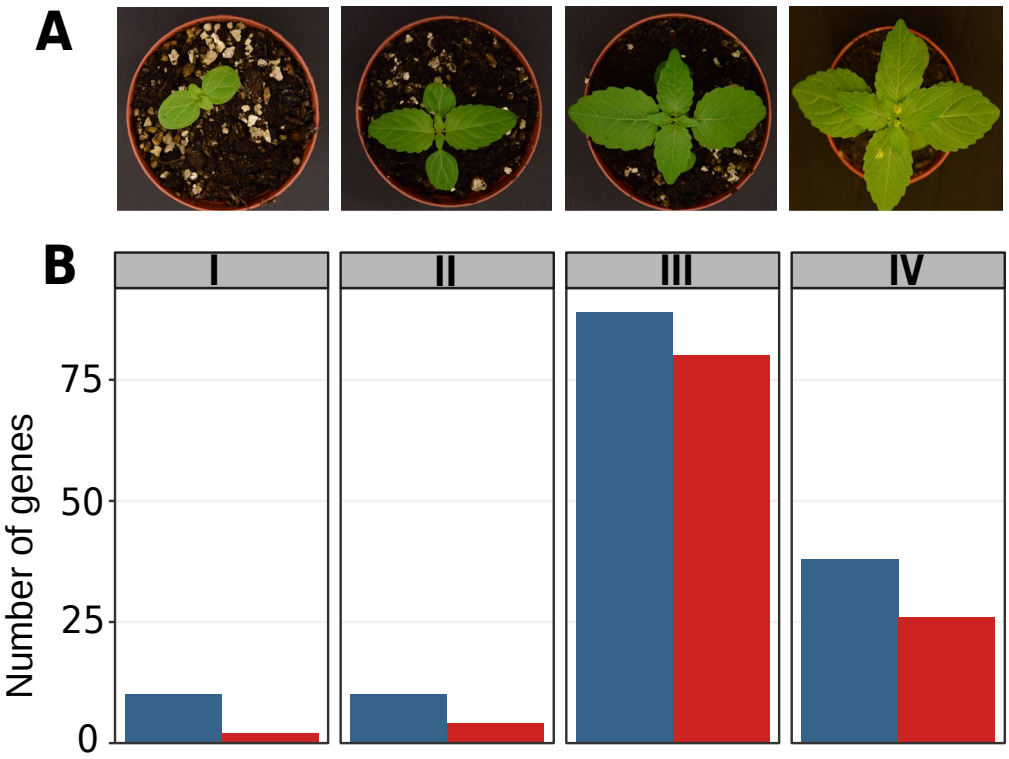

Figure 1. (a) Photos of typical (male) plant phenotype at each of the four growth stages sampled in Experiment 1. **(b)** Number of sex-biased genes found at each stage of development. Blue and red bars indicate male- and female-biased genes, respectively.

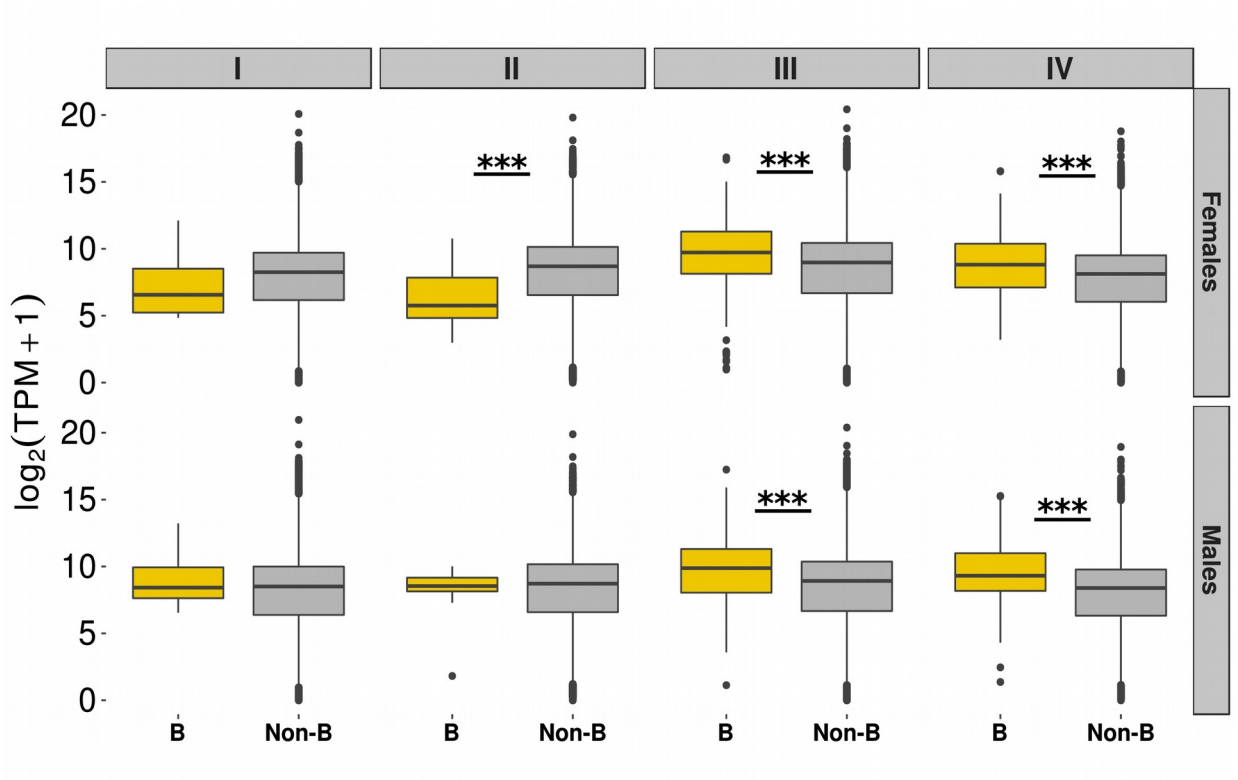

Figure 2. Boxplots of the expression level, measured as $\log_2(TPM+1)$, of the 972 sex-biased genes (yellow) and non-biased (gray) genes in males and females at the four developmental stages investigated in Experiment 1.

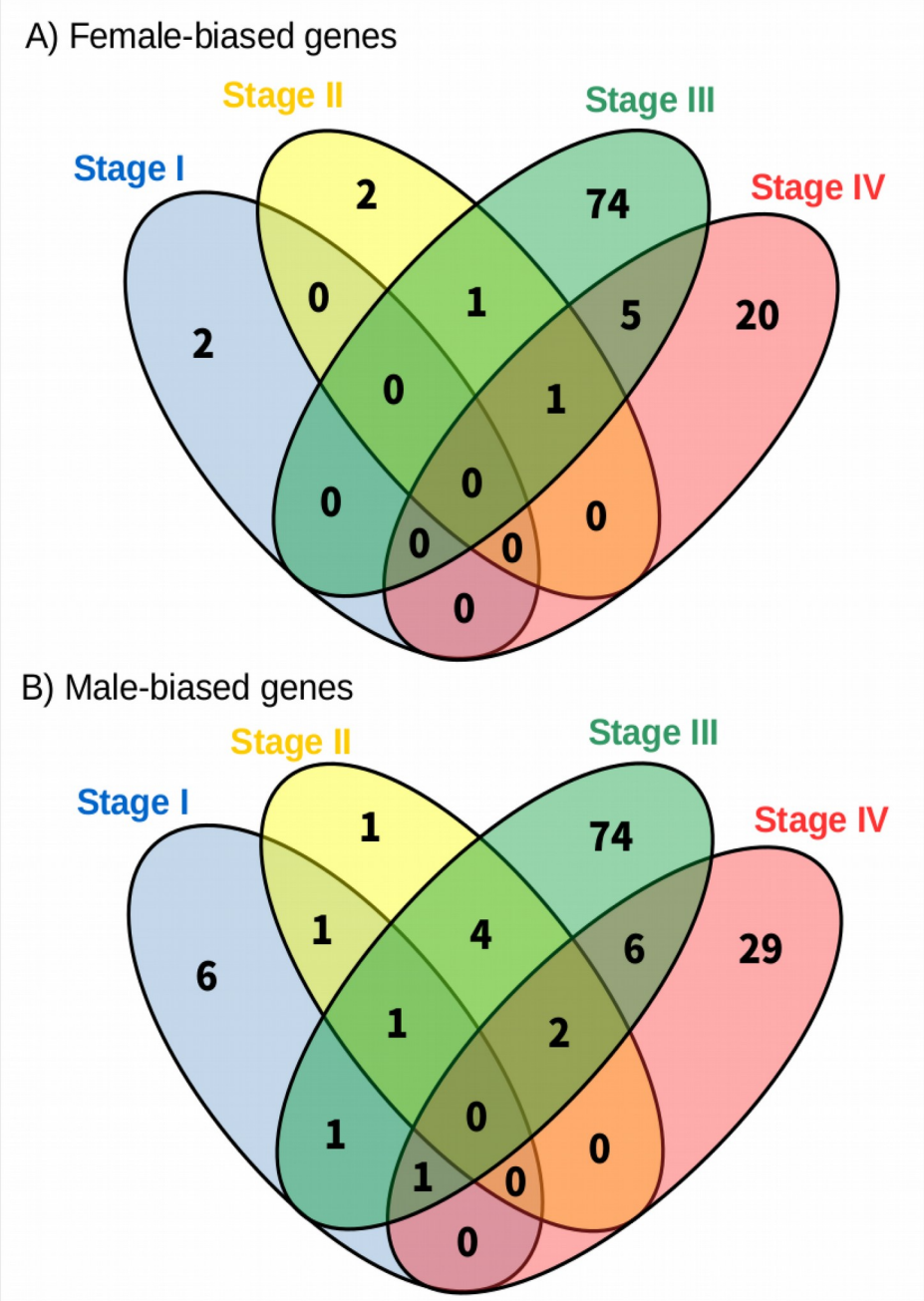

Figure 3. Venn diagrams representing the overlapping patterns of sex-biased genes across the four developmental stages investigated in Experiment 1. **(a)** Diagram for female-biased genes. **(b)** Diagram for male-biased genes.

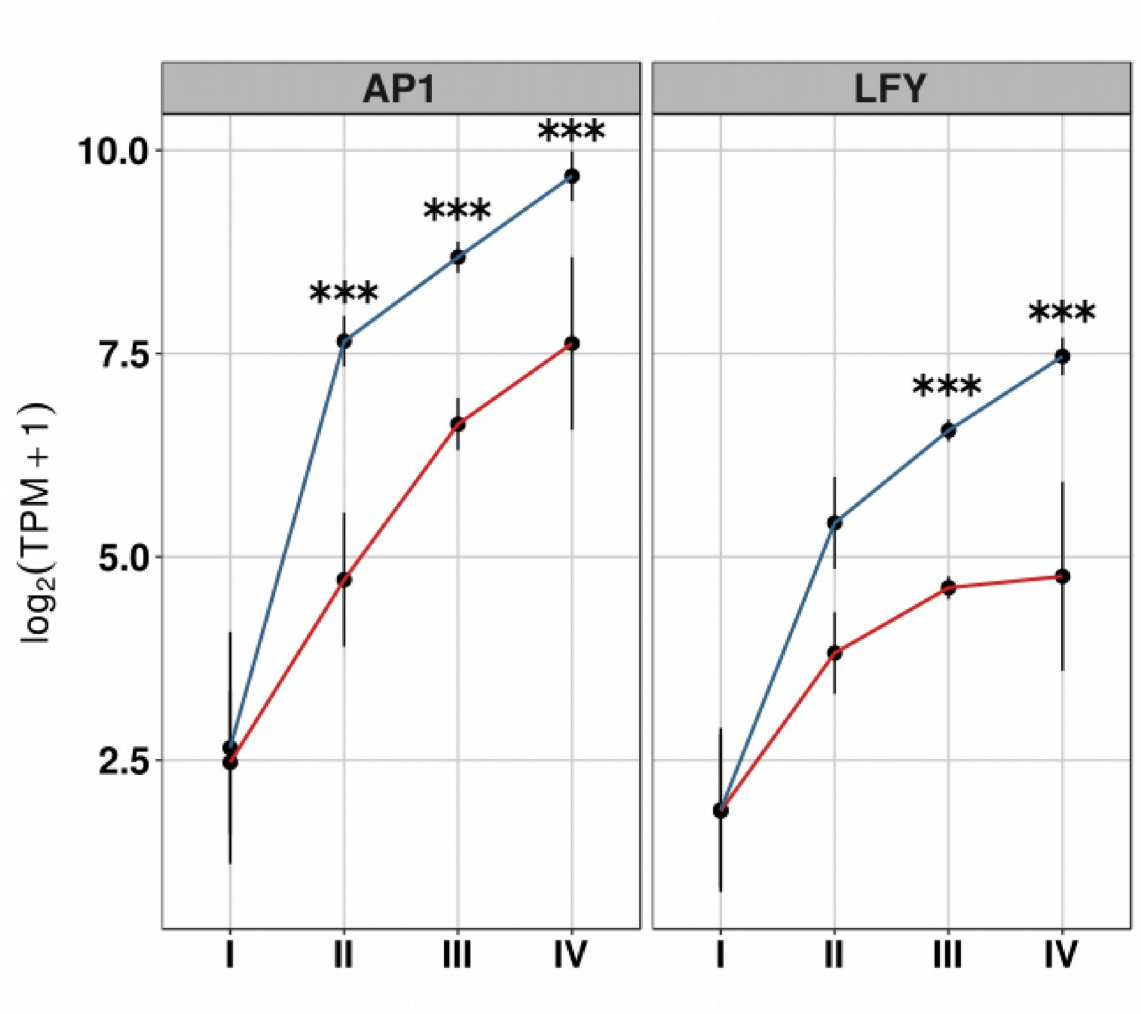

Figure 4. Tracking expression of *APETALA1* (AP1) and *LEAFY* (LFY) genes, calculated as $\log_2(TPM+1)$, across the four stages of development. Red and blue curves indicate the mean expression level in females and males, respectively. Black bars represent standard deviation. Stars indicate statistical significance of expression between sexes (** : $0.001 < P_{adj} < 0.05$; *** $P_{adj} < 0.001$).

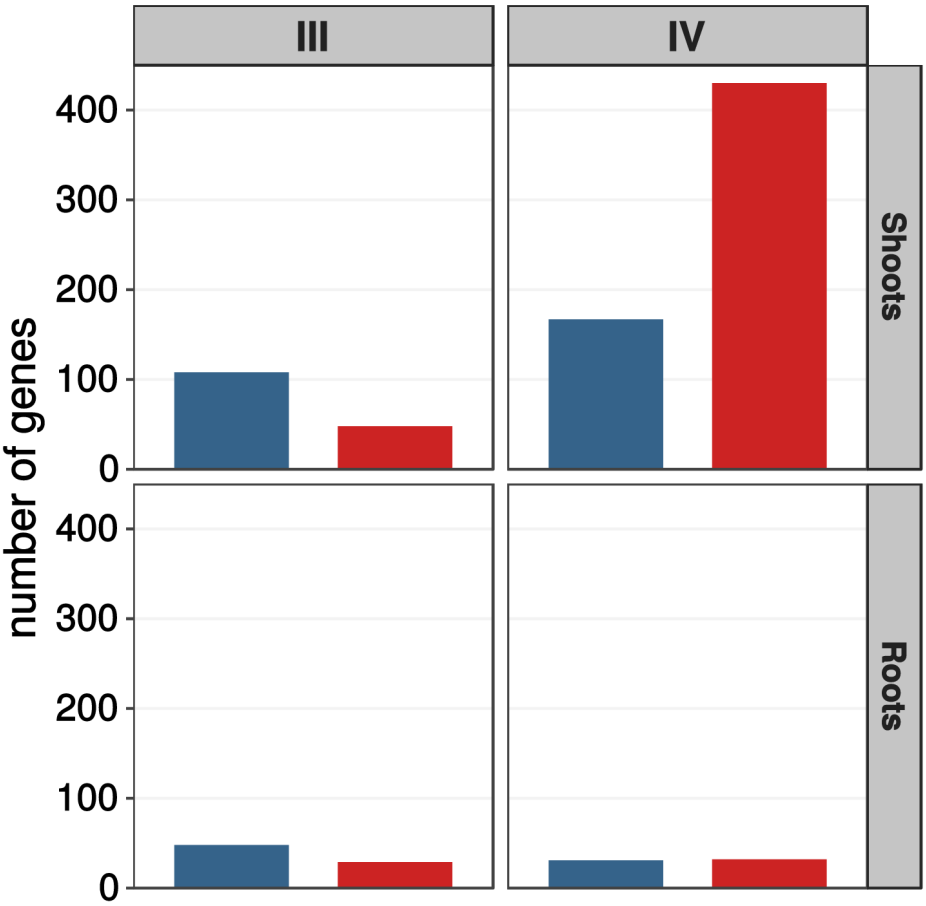

Figure 5. Number of sex-biased genes found at each of the two stages of development investigated in Experiment 2 (i.e., stages III and IV), in roots and shoots. Blue and red bars indicate male- and female-biased genes, respectively.

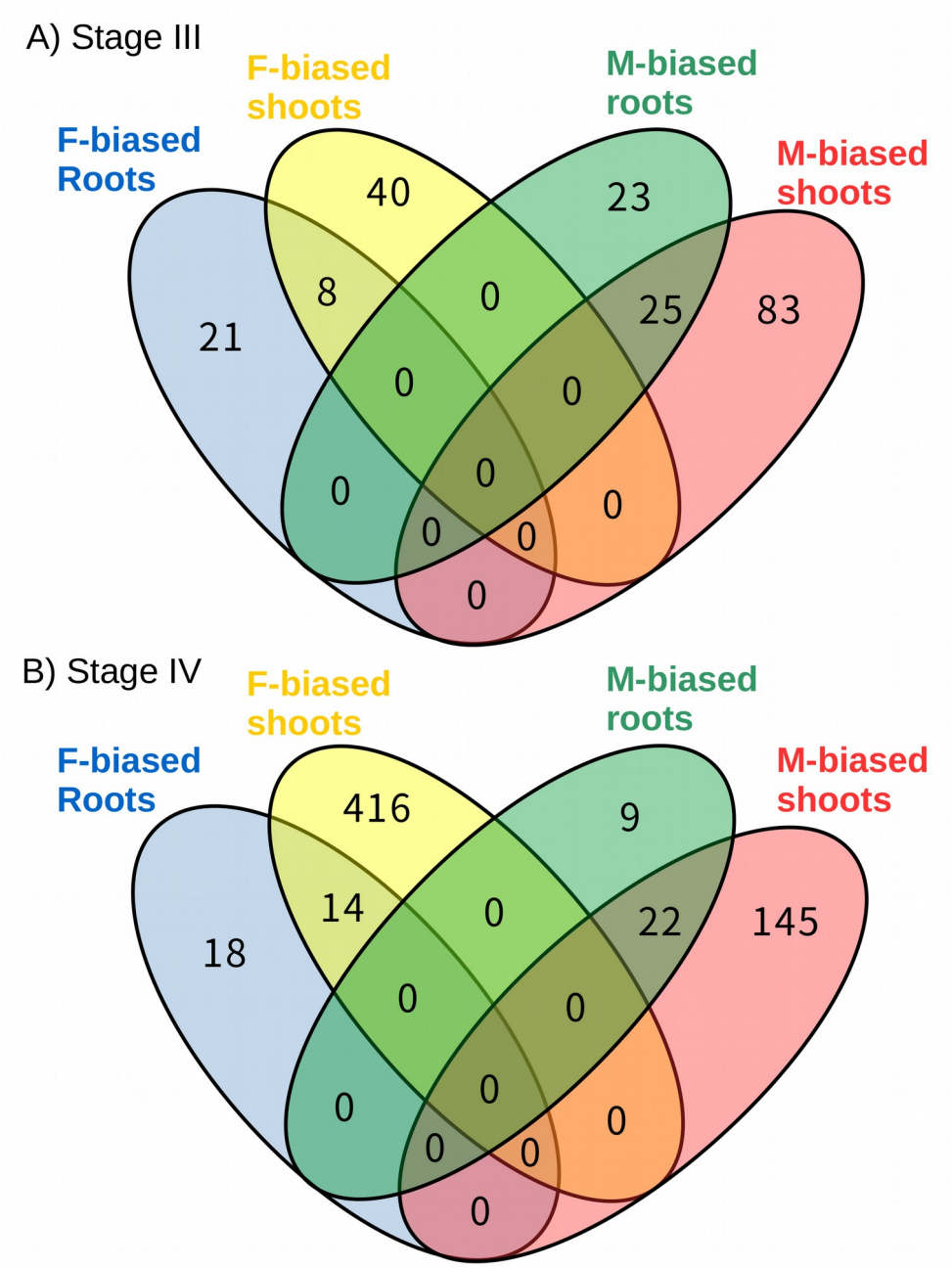

Figure 6. Venn diagrams representing the overlapping patterns of sex-biased genes across the tissues investigated in Experiment 2. **(a)** Overlapping patterns of sex-biased genes at stage III. **(b)** Overlapping patterns of sex-biased genes at stage IV. Blue and yellow circles show female-biased genes in roots and leaves, respectively. Green and red circles show male-biased genes in roots and leaves, respectively.

TABLES

Table 1. Sequencing pair-of-reads counts and alignment statistics from Kallisto (Bray *et al.*, 2016) for all sequenced *M. annua* pooled samples in Experiment 1.

Sample	Sex	Clean pair of reads	Pseudoaligned by Kallisto	Average fragment length
IF1	Female	48392886	11416134	179.18
IF2	Female	27567696	19947004	187.33
IF3	Female	26315347	19264460	182.02
IM1	Male	29756709	21958174	152.21
IM2	Male	27474394	20168852	160.8
IM3	Male	23652881	17058645	208.57
IIF1	Female	26509254	18825160	133.76
IIF2	Female	33723461	24710744	185.7
IIF3	Female	43801744	4742475	191.43
IIM1	Male	30426001	21950546	170.49
IIM2	Male	25780037	18811517	201.54
IIM3	Male	25484863	17062115	128.12
IIIF1	Female	26112620	19207373	171.00
IIIF2	Female	39384173	29018529	172.34
IIIF3	Female	50797364	37258280	168.35
IIIM1	Male	29882681	21775260	177.35
IIIM2	Male	38422934	25063807	172.64
IIIM3	Male	47479535	34919346	175.61
IVF1	Female	23954754	15209795	172.78
IVF2	Female	22388204	16395212	198.18
IVF3	Female	33797444	24366472	170.95
IVM1	Male	19689310	14162392	186.76
IVM2	Male	16902333	12056665	201.15
IVM3	Male	27916839	20215305	185.39

Table 2. Summary statistics of expression levels of sex-biased and non-biased genes in both males and females. Permutation *t*-test *P*-values are presented for 100,000 Monte-Carlo samples generated. Mean expression levels are given in terms of $\log_2(TPM+1)$.

Sex	**Statistics**	**I**	**II**	**III**	**IV**
Females	*P*	0.146	0.00318 ***	$<2.2\text{x}10^{-16}$ ***	$<2.2\text{x}10^{-16}$ ***
	Mean biased genes	8.48 ± 2.23	5.82 ± 2.70	9.36 ± 3.36	10.02 ± 2.69
	Mean non-biased genes	7.81 ± 2.95	8.18 ± 3.00	8.40 ± 3.12	8.12 ± 2.93
Males	*P*	0.0545	0.541	$<2.2\text{x}10^{-16}$ ***	$<2.2\text{x}10^{-16}$ ***
	Mean biased genes	8.96 ± 2.13	8.69 ± 1.11	10.07 ± 2.30	10.05 ± 2.80
	Mean non-biased genes	8.06 ± 2.98	8.21 ± 2.99	8.35 ± 3.10	7.86 ± 2.91

Table 3. Fisher's exact tests for GO-term enrichment (FDR < 0.05) of sex-biased genes. Significant enrichment was detected only at stages III and IV. Three representative enriched GO-terms are reported, when possible.

BIAS and Stage	GO-ID	GO Term	Category	FDR
M-biased III	GO:0004075	biotin carboxylase activity	F	1.95×10^{-5}
	GO:0003989	acetyl-CoA carboxylase activity	F	1.95×10^{-5}
	GO:0016885	ligase activity forming carbon-carbon bonds	F	2.54×10^{-5}
F-biased III	GO:0005982	Starch metabolic process	P	2.51×10^{-2}
	GO:0044435	Plastid part	C	2.51×10^{-2}
	GO:0009507	chloroplast	C	3.00×10^{-2}
M-biased IV	GO:0005515	protein binding	P	8.83×10^{-4}
	GO:0010468	regulation of gene expression	P	2.25×10^{-3}
	GO:0010582	floral meristem determinacy	P	8.89×10^{-3}

Table 4. Fisher's exact tests for GO-term enrichment (FDR < 0.05) of sex-biased genes. Three representative enriched GO-terms are reported when possible, for each tissue at both stages investigated.

Stage	Bias	Tissue	GO-ID	GO Term	FDR
Stage 3	**Male-bias**	**Leaves**	GO:0006119	Oxidative phosphorylation	1.42×10^{-2}
	Female-bias	**Roots**	GO:0097501	stress response to metal ion	1.95×10^{-2}
			GO:0061687	detoxification of inorganic compound	1.95×10^{-2}
			GO:1990169	stress response to copper ion	1.95×10^{-2}
		Leaves	GO:0000234	phosphoethanolamine N-methyltransferase activity	2.87×10^{-2}
			GO:0006656	phosphatidylcholine biosynthetic process	4.30×10^{-2}
			GO:0016642	oxidoreductase activity	4.30×10^{-2}
Stage 4	**Female-bias**	**Leaves**	GO:0015630	microtubule cytoskeleton	3.77×10^{-7}
			GO:0099080	supramolecular complex	3.69×10^{-6}
			GO:0044430	cytoskeletal part	2.89×10^{-6}

Table 5. *P-values* of Wilcoxon tests on *dNdS* between male-biased, female-biased and unbiased orthologs with *Mercurialis huetii*. Genes with *dS* > 0.0637341 and non-zero values for *dNdS* have been retained for the analysis.

dNdS	**Unbiased**	**Female-biased**	**Male-biased**	**Number of orthologs**	**Median**
Unbiased				4346	0.114
Female-biased	0.34			112	0.112
Male-biased	0.72	0.88		46	0.109
All biased	0.32	0.95	0.91	158	0.110

SUPPLEMENTARY INFORMATIONS

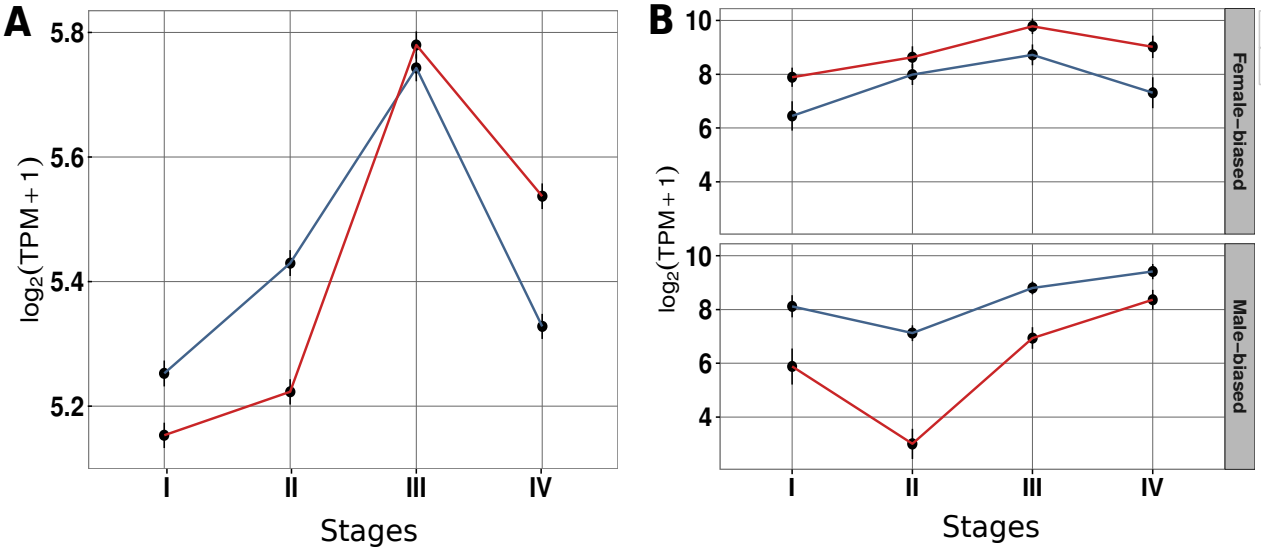

Figure S1. Tracking of the average expression of non-biased and sex-biased genes in males and females across the four developmental stages investigated in Experiment 1, measured in $\log_2$(TPM+1). Red and blues lines indicate the mean expression level in females and males, respectively. Black bars represent one standard deviation. (a) Mean expression of all expressed genes. (b) Mean expression of female-biased (upper) and male-biased (bottom) genes.

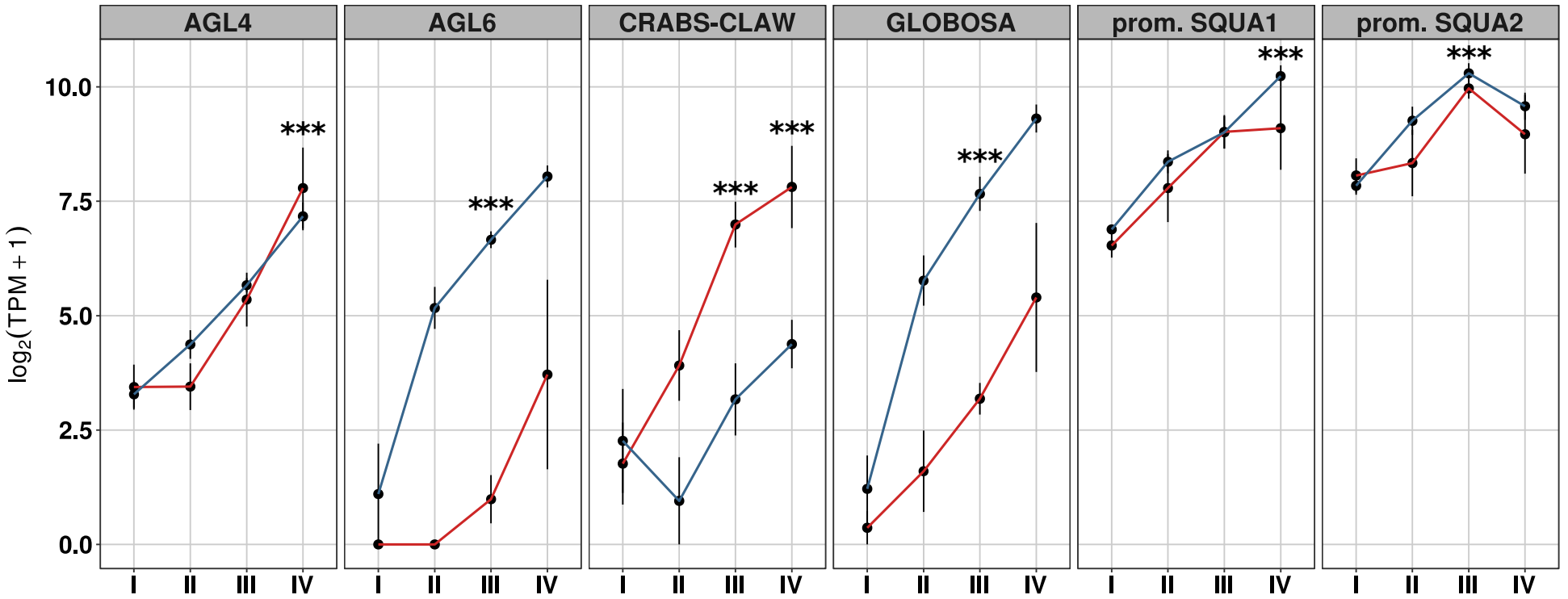

Figure S2. Tracking of the expression of extra developmental genes involved in determining the identity of floral organs, namely *AGAMOUS-like* 4 and *AGAMOUS-like* 6 (AGL4 and AGL6), *CRABS-CLAW*, *GLOBOSA*, and two promoters of *SQUAMOSA* (prom. SQUA1 and prom. SQUA2), across the four stages of development. Red and blues lines indicate the mean expression level in females and males, respectively. Black bars represent one standard deviation. Stars indicate statistically significant differences in expression between the sexes (** : $0.001 < P_{adj} < 0.05$; *** Padj < 0.001).

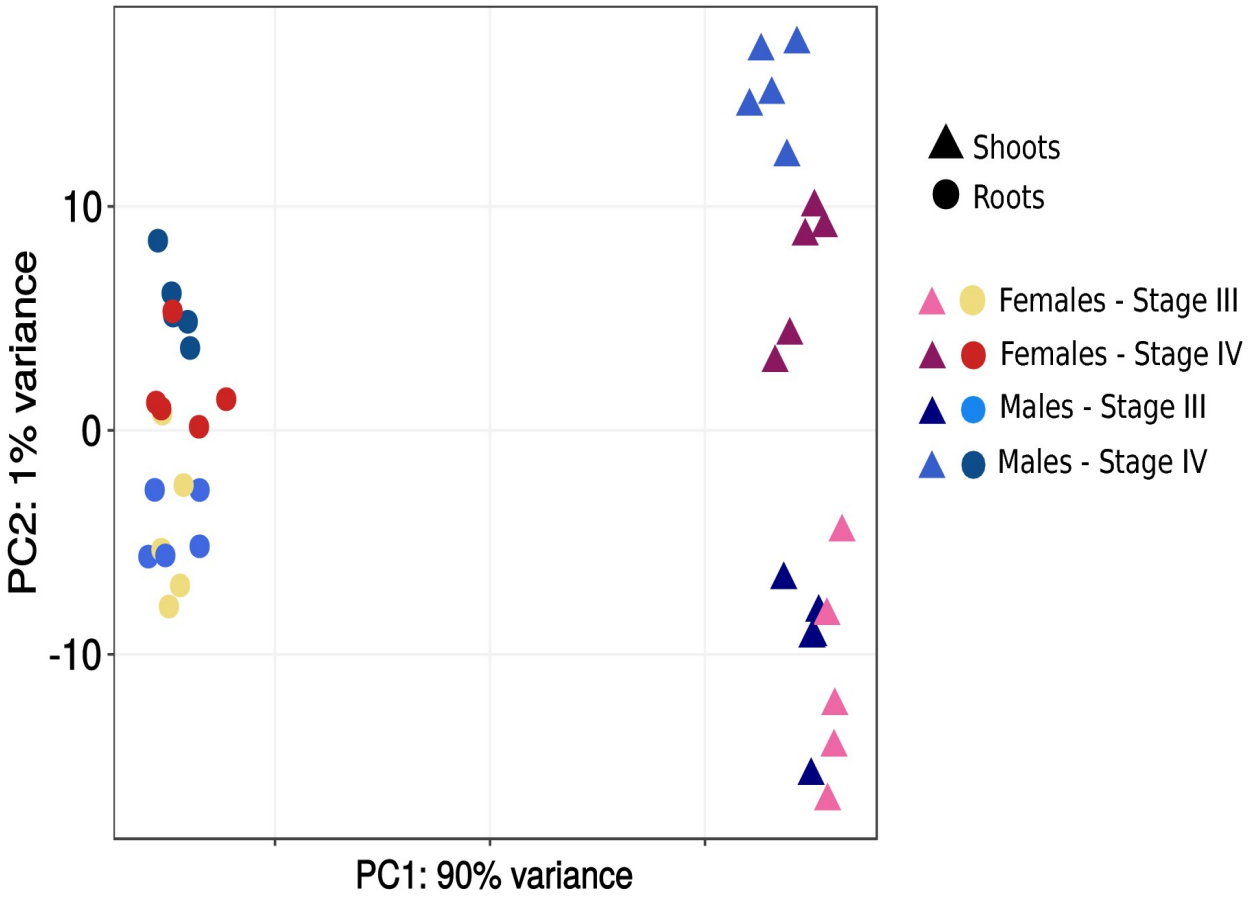

Figure S3. PCA plot of all samples studied in Experiment 2. Samples firstly clustered by tissue and then by stage within tissues.

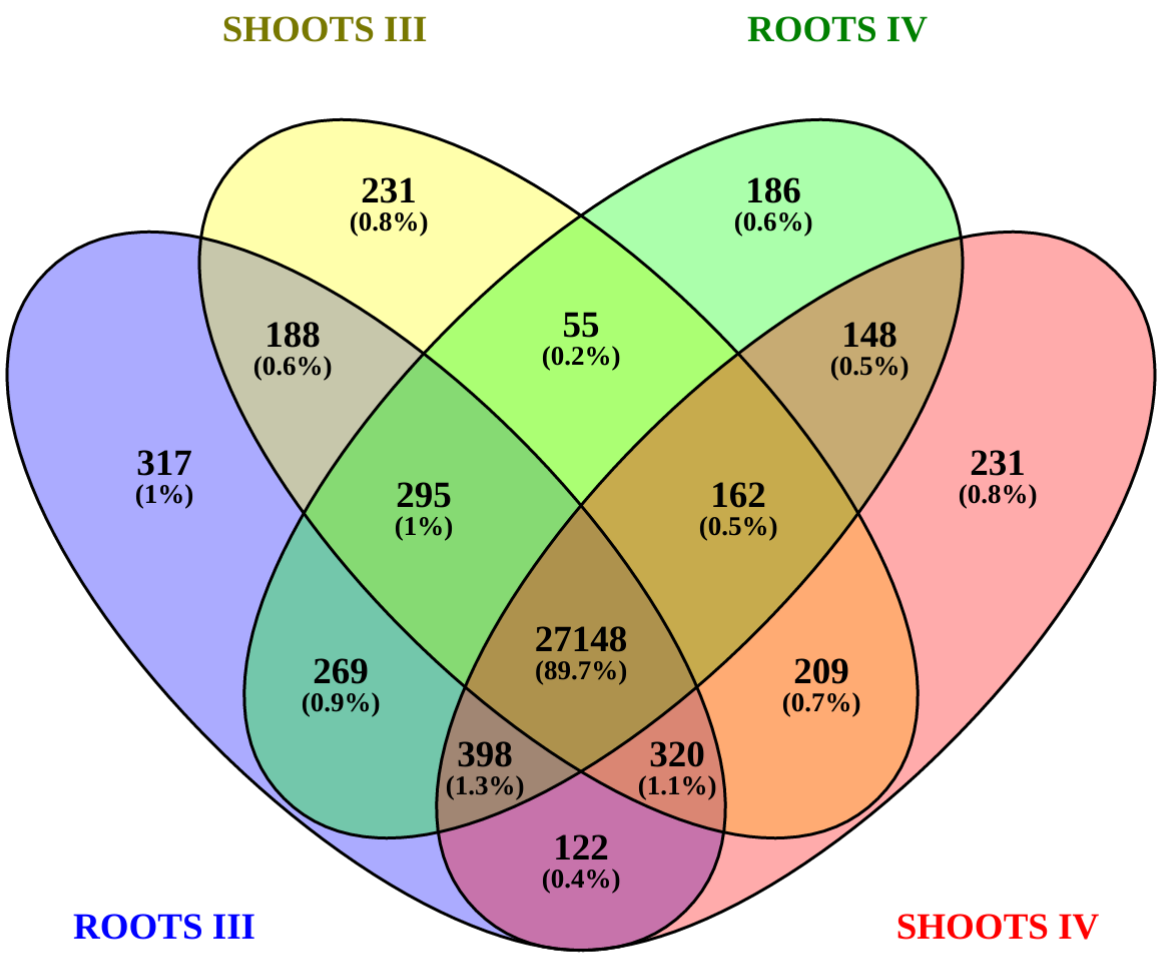

Figure S4. Venn diagram of all expressed genes across leaf and root tissues of Experiment 2.

Table S1 : Number of sex-specific and sex-biased genes as a proportion of total number of genes expressed at each stage of development. Sex-specific genes have on average across samples a null expression in one sex and above 10 TPM in the other sex.

Stage	Expressed genes	Direction of bias	Sex-specific (% expressed)	Sex-biased (% expressed)
I	27720	Male	26 (0.094 %)	23 (0.083 %)
		Female	23 (0.083 %)	20 (0.072 %)
II	27802	Male	39 (0.14 %)	15 (0.054 %)
		Female	21 (0.076 %)	2 (0.0072 %)
III	28279	Male	26 (0.092 %)	83 (0.29 %)
		Female	32 (0.11 %)	55 (0.19 %)
IV	28111	Male	15 (0.053 %)	52 (0.18 %)
		Female	35 (0.12 %)	28 (0.10 %)

Table S2. Number of sex-specific and sex-biased genes in Experiment 2, as a proportion of total number of genes expressed at each stage of development. Sex-specific genes had a null expression in one sex and above 10 TPM in the other sex, calculated as means across samples.

Stage	Tissue	Sex	Expressed genes	Sex-specific (% expressed)	Sex-biased (% expressed)
III	Roots	Male	28137	42 (0.15 %)	48 (0.17%)
	Roots	Female	28335	63 (0.22%)	29 (0.10%)
	Shoots	Male	28062	159 (0.57%)	108 (0.38%)
	Shoots	Female	27041	35 (0.13%)	48 (0.18%)
IV	Roots	Male	27802	62 (0.22%)	31 (0.11%)
	Roots	Female	27745	65 (0.23%)	32 (0.12%)
	Shoots	Male	27858	103 (0.37%)	167 (0.60%)
	Shoots	Female	27690	80 (0.29%)	430 (1.55%)

- CHAPTER 3 -

A functional perspective of sex inconstancy in dioecious *Mercurialis annua*: implications for sexual-system evolution

Guillaume Cossard and John R. Pannell

Introduction

The great majority of flowering plants are hermaphroditic, but separate sexes (dioecy) have evolved frequently and are found in about half of the approximately 400 angiosperm families (Renner and Ricklefs 1995; Renner 2014). In contrast to most animals with separate sexes, many dioecious plants show an expression of sex or gender that is not entirely constant: males occasionally produce fruits, and females occasionally produce flowers with pollen, and may thus sire progeny as fathers. Such individuals have been referred to as 'leaky' (e.g., (Humeau *et al.* 1999; Anderson and Bernardello 2006; Venkatasamy *et al.* 2007; Wang *et al.* 2014) or 'inconstant' in their sex expression (Lloyd 1975a; b; Ehlers and Bataillon 2007; Crossman and Charlesworth 2014). A common pattern in such species is that it is usually the males that show leaky sex expression, not the females (Westergaard 1958; Charlesworth and Charlesworth 1978; Delph and Wolf 2005; Ehlers and Bataillon 2007). Indeed, in a review of leaky sex expression in plants, Ehlers and Bataillon (2007) found that males were the leaky sex in 78% of cases in which sex inconstancy had been observed. Their survey did reveal a minority of cases in which females produced some pollen, but in all of these cases both sexes were found to be inconstant.

Sex inconstancy has been reported for a number of species since Ehlers and Bataillon's (2007) review, and several cases published previously were not included in their review. In most of these species, again only males showed inconstancy. For instance, Strittmatter et al. (2008) recorded male inconstancy stemming from the development of functional carpels in male flowers of *Consolea moniliformis* (Cactaceae), and they similarly reported leaky males in natural populations of the related species *C. corallicola, C. picardae and C. spinosissima*. In the dioecious tropical tree *Jacaratia mexicana* (Caricaceae), fruiting males were observed at a rate of up to 45% in some populations (Aguirre *et al.* 2007). Sawyer and Anderson (2000) confirmed cryptic dioecy in *Deprea paneroi* (Solanaceae), *with a few* males that exceptionally produced fruit. Lazarte and Palser (1979) recorded the presence of pistils in some male flowers of *Asparagus officinalis* (Asparagaceae) *and an associated production of* up to about a dozen fruits per male.

Several studies not mentioned by Ehlers and Battaillon (2007) document inconstancy in both sexes. Venkatasamy et al. (2007) recorded leakiness in *Diospyros egrettarum* (Ebenaceae), in which 2% of individuals produced flowers of both sexes and viable seeds, evidently with no prevalence in one sex or the other. Similarly, Wang et al. (2014) confirmed leakiness in the dioecious species *Eurya obtusifolia* (Pentaphylaceae), reporting individuals with hermaphroditic flowers capable of self-fertilization (Wang *et al.* 2014). Finally, Schlessman et al. (1990) reported low levels of inconstancy in both males and females of *Polyscias pancheri* (Araliaceae). As with the studies reviewed by

Ehlers and Bataillon (2007), none of these additional cases show a prevalence of inconstant females.

The prevalence of inconstancy in males versus females of a species likely reflects its evolutionary history (Charlesworth and Charlesworth 1978; Lloyd 1980a; Delph and Wolf 2005). Thus, the expression of inconstancy only in males may reflect a gynodioecious path towards dioecy, where females are the expression of a major male-sterility mutation and males have evolved through an increasing emphasis of their male function, retaining a residual female function under certain circumstances (Charlesworth and Charlesworth 1978; Lloyd 1980a; Delph and Lloyd 1991; Spigler and Ashman 2011). Similarly, the presence of leakiness in both sexes has been interpreted as a signature of the evolution of separate sexes via the 'monoecy-paradioecy' pathway (Freeman *et al.* 1997; Renner and Won 2001; Dorken and Barrett 2003), where two classes of male- and female-biased hermaphrodites gradually evolve towards increasing maleness and femaleness, respectively, so that inconstancy represents an unfinished trajectory towards the full separation of sexual functions (Lloyd 1980a; Renner and Won 2001). The prevalence of male inconstancy is consistent with the important role that gynodioecy has likely played in the evolution of dioecy. In contrast, there appears to be a complete dearth of species in which females are the only inconstant sex. Indeed, to our knowledge, in all species in which inconstant females have been reported, inconstant males also occur. This apparent lack of female-only sex inconstancy in plants is consistent with the negligible role that androdioecy (the co-occurrence of males with hermaphrodites) is thought to have played in the evolution of dioecy compared with gynodioecy (Charlesworth and Charlesworth 1978; Charlesworth 1984).

The rarity of androdioecy in flowering plants (Renner and Ricklefs 1995; Renner 2014) is predicted by models that show that female-sterility mutations should spread among hermaphrodites under much more stringent conditions than male-sterility mutations, especially if the hermaphrodites are partially self-fertilizing (Charlesworth and Charlesworth 1978). Indeed, the few known cases of androdioecy that do exist appear to have evolved not from hermaphroditism via the spread of a female-sterility mutation, but rather through the breakdown of dioecy upon the selection of leaky females and an evolved increase over time of the rate and level of expression of a male function (Pannell 2000, 2002, 2008; Wolf and Takebayashi 2004). Because this model assumes the presence in the population of leaky females upon which selection might act, we might expect to find similar leaky females in dioecious populations related to those that have evolved androdioecy. A finding of sex inconstancy only in females of such species would point to a history of androdioecy and the spread of a female-sterility mutation in the evolution of the dioecious lineage. Alternatively, if

inconstant females co-occurred with inconstant males in the population, androdioecy as the expression of a major female-sterility mutation would be less likely. Either way, assessment of the distribution of leaky sex expression in lineages associated with known androdioecy would begin to fill an important empirical lacuna in our knowledge of the potential role of sex inconstancy in linking hermaphroditism with dioecy in flowering plants.

Here, we analyze the distribution of sex inconstancy in diploid populations of the wind-pollinated annual dioecious herb, *Mercurialis annua*. The *M. annua* species complex is remarkable for its wide variation in sexual systems, including dioecy and monoecy in diploid and tetraploid populations, respectively, but also androdioecy in many hexaploid populations (Durand 1963; Durand and Durand 1992; Pannell 1997d; Pannell *et al.* 2004, 2008; Obbard *et al.* 2006; Dorken and Pannell 2009). In its dioecious populations, Yampolsky (1919, 1920) noted the presence of 'intergrades' in both sexes (evidently individuals showing inconstancy), a feature also recorded by Durand (1963). However, neither of these authors provided a quantitative assessment of inconstancy, nor did they situate inconstant sex expression within the context of sexual-system variation in the genus more generally. In this article, we build substantively on Yampolsky's observations by placing variation in sex expression in *M. annua* into the quantitative functional framework introduced by Lloyd (1980b) and widely adopted in the literature on the distribution of gender in both monomorphic and dimorphic populations (e.g., (Schlessman *et al.* 1990; Delph and Lloyd 1991; Barrett 1992; Sarkissian *et al.* 2001; Delph and Wolf 2005; Pannell 2005). Our quantitative analysis of sex inconstancy in dioecious *M. annua* throws further light on the likely evolutionary paths that have given rise to the remarkable variation displayed among different lineages within the species complex. Previous work on sexual-system evolution in *M. annua* has pointed to the potential role played by polyploid hybridization for the origin of males in its hexaploid populations, specifically through the introgression of a Y chromosome from a dioecious lineage into a monoecious lineage (Obbard *et al*. 2006). Our quantitative analysis presented here emphasizes the important role that phenotypic variation in sex expression has likely played in sexual-system transitions, irrespective of ploidy.

Lloyd's (1980b) quantitative framework for describing the distribution of gender in plant populations places all individuals on a sex-allocation continuum between fully canalized males and fully canalized females, including inconstant individuals and hermaphrodites with more equal sex allocation. Specifically, if g_i and p_i are the investments made in female and male function by the ith individual of a population, then the quantitative femaleness of individual i is given by $G_i = g_i/(g_i + Ep_i)$, where $E = \Sigma g_j / \Sigma p_j$ is an equivalence factor, with the sums taken over all individuals of the

population, that ensures equal gene transmission between male and female functions at the population level (Lloyd, 1980b). Because G_i is typically computed for a reasonable sample of individuals in a population of interest, its distribution implicitly accounts for both the degree to which each individual is male versus female, and the frequency or rate of individual with that gender strategy. We adopt a similar approach for an analysis of sex inconstancy, except that we explicitly compute both the rate and the degree of inconstancy for the populations sampled. Inconstant or leaky sex expression in plants has typically been discussed in verbal rather than in quantitative terms. For example, although Ehlers and Bataillon (2007) set a 5% threshold for the proportion of inconstant individuals for inclusion of a population in their table of observed cases of sex inconstancy, they implied no measure that might quantify the degree to which a given individual might express the opposite gender function. Indeed, data that might allow this calculation are rarely reported in the literature (but see (Freeman and McArthur 1984; Delph 1990; Huff and Wu 1992). We show that Lloyd's quantitative measure of gender is particularly useful for measuring the degree of inconstancy. Specifically, we measure an individual's degree of inconstancy by calibrating its allocation of resources to the inconstant sex against the reproductive effort made by constant individuals of that sex (see Materials and Methods).

Materials and methods

Study species, study populations, and sampling

Mercurialis annua is a wind-pollinated annual herb in the family Euphorbiaceae. The genus *Mercurialis* is almost exclusively dioecious, and dioecy is likely the ancestral sexual system in the genus (Obbard *et al.* 2006). Dioecious populations, which are exclusively diploid, are distributed widely around the Mediterranean Basin and throughout central and western Europe, from Israel through eastern, central and western Europe, and into the northern Iberian Peninsula (Durand 1963; Durand and Durand 1992; Pannell *et al.* 2004). Females produce their flowers in their leaf axils, whereas males bear their flowers on long, erect inflorescence stalks (peduncles) (Pannell 1997a; d; Pannell *et al.* 2008). Inconstant males produce fruits in their leaf axils or along their peduncles, but inconstant females lack the typical male inflorescence morphology and produce staminate flowers in their leaf axils.

We assessed patterns of sex inconstancy in three common-garden populations, which we established over two consecutive years (2012 and 2014) from seeds sampled from a pool of 25 populations of dioecious *M. annua* across its range in Europe that we have used as a base for other studies on the species (Labouche and Pannell 2016). This pool of genotypes is genetically diverse and likely

represents much of the variation in sex expression observed across the species' range (G. Cossard and J.R. Pannell, personal observation). We established each common garden by transplanting 180 seedlings from seed trays into a dense hexagon of pots on the campus of the University of Lausanne, Switzerland; populations had a 1:1 sex ratio. Soil was enriched with slow-release fertilizer (10g/50L of soil).

After seven weeks of growth, 50 males and 50 females from each replicate were randomly harvested and assessed for their expression of sex inconstancy. We recorded the sex, dry-mass and height of all plants in the common gardens. At this stage of growth, plants were all sexually mature and females were all producing mature fruits. Given the high density of our experimental populations and the fact that *M. annua* is wind-pollinated, pollen was abundant in the common gardens, so that seed set was probably not pollen-limited (Hesse and Pannell 2011a; Labouche and Pannell 2016); thus seed production closely reflects female flower production (Hesse and Pannell 2011a). We collected and weighed male flowers present on the plant at the time of sampling, and similarly assessed female allocation by weighing the total production of mature seeds at the time of harvest. Fruits of *M. annua* are usually two-seeded (Hesse and Pannell 2011a), so that we estimated the number of successful female flowers produced by focal females as half the number of seeds produced by an individual. In 2012, we recorded the numbers of seeds produced by each male and female, as well as the numbers of male flowers produced by females. In 2014, we recorded the number of flowers of both sexes produced by both males and females. We then weighed the total male flower and seed dry biomass. For some individuals, male flower production was too low to be measured by our balance (which was precise to 0.00001 g); in these cases, we multiplied the calibrated average weight of a single male flower (based on 15 samples of 50 male flowers each) by the number of flowers counted.

Quantifying sex inconstancy in *M. annua*

We define: (1) the 'rate of inconstancy' as the proportion of plants of a particular sex showing any production of flowers of the other gender (r_f and r_m for inconstant females and males, respectively); and (2) the 'degree of inconstancy', *d*, as the extent to which an inconstant individual of one sex allocates resources to the other gender, relative to constant plants of that other sex in the population. In other words, *d* measures the extent to which inconstancy in sex expression has taken an individual from zero to one on a quantitative scale of their normalized inconstant reproductive effort (see Figure 1). To account for the fact that allocation to reproduction scales roughly linearly with plant size in many species (Klinkhamer *et al.* 1997), and to allow a fair comparison between males and females in species that show sexual size dimorphism, e.g., with males smaller than females, as

is the case for *M. annua* (Harris and Pannell 2008; Sánchez-Vilas and Pannell 2010, 2011; Sánchez-Vilas *et al.* 2011), we measured the degree of inconstancy in terms of a normalized male or female reproductive effort, i.e., in terms of mass allocated to that gender divided by the plant's total above-ground biomass. The degree of inconstancy, *d*, can then be computed for a given inconstant female as $d_f = \square_f / A$, where $\square_f$ is its male reproductive effort (i.e., the biomass allocated to male function, divided by its total above-ground biomass, and *A* is the reproductive effort of an average constant male in the population (Figure 1d). d_f effectively measures the extent to which a female has moved along the continuum from 0 to *A* in expressing a male function; typically, this increment would be << *A* (Figure 1d). Similarly, let $d_m = \square_m / G$, where *G* is the reproductive effort of an average female in the population (i.e., the biomass of female flowers and fruits divided by above-ground biomass) (see Figure 1e).

We computed the rate of inconstancy, *r*, as the frequency of individuals for each respective sex that produced at least one flower of the opposite gender. We computed *d* from our data in two ways: as mean across all inconstant individuals of a particular sex, d_{inc}; and as an average measure computed over all individuals of that sex, including those that were not inconstant, d_{all}. While d_{inc} permits to compare inconstant males and females directly, d_{all} indicates the overall degree of inconstancy for each gender at the population level. Because data allowing an estimate of *A* were not collected in 2012, we calculated *d* for 2012 using the average value of *A* across populations as estimated in 2014. We used Kolmogorov-Smirnov tests to compare *d* between males and females. We similarly computed *A*, *G*, □ and *d* in terms of estimates of flower number production (A_n and G_n in Table1).

To analyze the effects of sex, dry biomass, population and year of the growth of plants on the presence or absence of minority sex flowers on an individual, we used a zero-hurdle model (Zuur *et al.* 2009). We then used a count model to assess whether the above variables had an effect on the number of flowers produced in the class of inconstant individuals (Zuur *et al.* 2009), using a negative binomial distribution to model the count data. Results are presented for the minimum models for each part of the hurdle model.

Results

Our analysis of the rate and degree of sex inconstancy in *M. annua* revealed strong variation in patterns of growth and reproductive allocation across replicate populations and, particularly, across the two years of our study (see Table 1). Because of the differences observed between years, we present and analyze our results for each of the two years separately.

Patterns of reproductive allocation for constant males and females

Averaging across the three replicate populations raised in 2014, the number of flowers produced was 16.71 times higher for constant (non-leaky) males than for constant (non-leaky) females (Table 1, data non-available for 2012). Taking account of the fact that *M. annua* shows sexual size dimorphism (Harris and Pannell, 2008), and that females in our study were on average 1.33 times larger than males, males produced 22.25 times more flowers per unit of above-ground biomass than did females. In terms of reproductive biomass, males invested 2.39 times more than did females in absolute terms, or 3.19 times in terms corrected for the larger total biomass of females.

We used the reproductive effort of constant males and females to determine the value of A and G for each replicate population; recall that A effectively corresponds to the maximum prospective reproductive effort in terms of male flower biomass that an inconstant female could achieve by allocating all its reproductive resources to its male function, i.e., by effectively becoming a male, and G corresponds to the equivalent term for inconstant males. We found that $A = 0.14$ in 2014, and $G = 0.073$ and 0.044 in 2012 and 2014, respectively (recall that we did not record all data needed to estimate A in 2012; Table 1). These values are used below in our calculations of the normalized inconstant reproductive effort, d.

Rate of inconstancy in males and females

The rate of inconstancy, r, was much higher in females than males and differed markedly between years. Across all three replicate populations, the values were 0.013 and 0.36 for inconstant males and females, respectively, in 2012, and 0.047 and 0.16 in 2014. The frequency of inconstant females was thus 27 times higher than that of inconstant males in 2012, but only 3 times higher in 2014 (Table 1); the difference between years is highly significant, with $P = 8.37 \times 10^{-5}$. Different populations did not differ significantly within years, nor did biomass differences affect the rate of inconstancy between populations (Table 2). Inconstant males were on average smaller than inconstant females, similar to the difference between constant males and females (above), but not significantly so, probably because of the small numbers of inconstant males.

Degree of inconstancy in males and females

The number of flowers of the opposite sex produced by inconstant males and inconstant females varied significantly across years ($P = 0.0057$; Table 2), but not among populations within years. The average biomass of constant and inconstant males and females individuals were not significantly different within years (data not shown). Similarly there were no significant differences in the

production of seeds or male flowers between inconstant and canalized individuals in either females or males, respectively (data not shown).

Inconstant females produced 2.12 and 1.30 times more flowers of the opposite gender than did inconstant males in 2012 and 2014, respectively (Table 1b, Figure 2). In terms of the biomass of reproductive allocation, inconstant females actually invested 2.12 times more than inconstant males in flowers of the opposite gender in 2012, but 5.95 times less in 2014, highlighting the high variability of sex allocation and inconstancy in *M. annua.* Inconstancy in terms of biomass, normalized against the estimated maximum allocation achievable for each inconstant gender (*A* for inconstant females and *G* for inconstant males), was higher for males than females, i.e., inconstant males tended to occupy a position further along the axis of relative femaleness than that occupied by inconstant females along the corresponding axis of relative maleness (see Figure 1). Overall, the normalized inconstant reproductive effort, *d*, for males was 20.4 times that of females in 2014. In other words, although there were many more inconstant females than inconstant males in our populations, when males were inconstant, they tended to be more so than females.

Discussion

Variation in sex expression in males and females of dioecious *Mercurialis annua*

Our study found substantial expression of sex inconstancy in dioecious *Mercurialis annua*, confirming previous observations by (Yampolsky 1919, 1920) and others (Vries 1901 p. 572; Durand and Durand 1992). Although expression of sex inconstancy in *M. annua* was similar across the three common gardens of our study, it varied strongly across the two years of our study, both in terms of the rate of inconstancy and its degree. Given than our replicate populations were drawn from a single base population, these differences can be attributed largely to plasticity. Plastic variation in sex inconstancy also occurs in polyploid *M. annua*, in which individuals that are only female in the field express both sex functions under glasshouse conditions (Pannell 1997d), and where hermaphrodites generally vary as a function of factors such as density, shading, and soil quality (Pannell 1997b; Hesse and Pannell 2011a; Sánchez-Vilas and Pannell 2014).

Lability and plasticity are probably quite general phenomena in dioecious plants that show sex inconstancy (Bawa 1980; Delph and Wolf 2005; Ehlers and Bataillon 2007), though the factors to which plants are sensitive in their sex expression have rarely been investigated. We do not know to which environmental factor dioecious *M. annua* responded in its sex inconstancy in our study. Yampolsky (1919) noted that sex inconstancy was more prevalent in plants damaged by cutting, a

stress we did not manipulate. Plasticity in the sex allocation of hermaphrodites in polyploid populations of *M. annua* has been attributed to environmental differences in shade (Pannell 1997b), nutrient availability (Hesse and Pannell 2011b; Sánchez-Vilas and Pannell 2014), and competition (Hesse and Pannell 2011c). Although we attempted to keep these variables uniform over our common gardens, unmeasured variation in local interactions between and within the plots may have affected plant phenotypes.

We measured both the rate of inconstancy in males and females of *M. annua*, *r*, as well as its degree, *d*. We found that females were more frequently inconstant than males, i.e., they had a greater *r*. This observation contrasts with the pattern reported in plants more generally (Ehlers and Bataillon 2007). When only one sex of a plant population shows leaky sex expression, it is almost always the male; moreover, although patterns have not been reported in sufficient detail to draw conclusions about the more inconstant gender when both sexes are inconstant, it would appear that greater inconstancy in females is probably unusual (Delph and Wolf 2005; Ehlers and Bataillon 2007; and see Introduction). We computed *d* as the normalized degree of inconstancy on a scale from zero to one, where zero represents no leaky expression and one represents a degree of investment in the opposite sex represented by that made by non-leaky individuals of that sex. Use of *d* both recognizes the frequency dependence of functional gender (Lloyd 1980b), but because it is a unitless, relative, measure, it also sidesteps the often-difficult question of which currency to use when measuring allocation to sex (Bazzaz *et al.* 1987, 2000, Reekie and Bazzaz 1987a; b; Gleeson and Tilman 1992). In both years of our study, inconstant males of *M. annua* had a greater *d* than inconstant females. In other words, inconstant males moved further towards complete femaleness than inconstant females did towards complete maleness. This conclusion continued to hold in 2014 (though not in 2012, when inconstant males were particularly rare). Here, we computed *d* as an average over all males or females in the population, i.e., d_{all}, which includes both inconstant and constant individuals (essentially, d_{all} is a measure of the average prospective gender of each respective sex class; Lloyd, 1980b). Thus, while females were more frequently inconstant in our study, males showed a greater degree of inconstancy and, taking into account the lower frequency of males that were inconstant, males on average were functionally more female than females were male.

Although leakiness appears to be frequent in *M. annua*, at least under some conditions, our estimates of d_{all} in large outcrossing populations suggest that it only slighty shifts in the functional gender of the two major sex classes away from complete gender separation. If we assume random

union of pollen and ovules in dense populations in which mates are abundant, which seems plausible for a wind-pollinated species, inconstant males and females should produce or sire only 0.17 % and 0.049 %, respectively, of the seeds produced under the conditions of our experiment in 2014. Thus, although the frequent leakiness expressed by individuals of *M. annua* might appear to qualify the species as effectively 'subdioecious', the low estimates of *d* imply that males and females are functionally almost completely canalized in their gender, unless mates are limited (see below). Similarly, although sex inconstancy in *M. annua* is strongly asymmetrical, with females substantially leakier than males, the species is not functionally androdioecious or subandrodioecious (Pannell 2005), because d_{all} is so low for both sexes. This interpretation is consistent with observations that the sex ratio in dioecious *M. annua* rarely deviates from 1:1 (Russell and Pannell 2015): if leaky females contribute substantially to the male function of the population, their frequency should exceed that of males, as in androdioecious populations of hexaploid *M. annua* (Pannell 1997b; c).

Functional implications of leaky sex expression for *M. annua*

Given that inconstant sex expression in *M. annua* shifts the gender of males and females from complete separation of the sexes by so little, what might be the functional implications of leakiness? In dense populations, males of *M. annua* enjoy a substantial siring advantage by dispersing their pollen from their erect 'pedunculate' inflorescences (Eppley and Pannell 2007). Given that seed production by inconstant males likely diverts resources away from pollen production and dispersal for which they are adapted, we might suppose that sex-inconstant males pay a net cost in dense populations in which there are substantial opportunities for outcrossing. In contrast with males, leaky females disperse their pollen from female-like inflorescences in the leaf axils, so that pollen grains produced by females will probably be less successful in reaching stigmas of other plants than those produced by males. Indeed, Eppley and Pannell (2007) showed that pollen grains dispersed from axillary inflorescences typical of leaky females are about 40% less likely to be successful in siring ovules than pollen grains dispersed by males. Dioecy in *M. annua* is likely maintained by an overall acceleration of the male and/or female fitness gain curves (the fitness set), underpinned by sexually dimorphic inflorescence and growth traits (Charnov *et al.* 1976; Campbell 2000; Pannell *et al.* 2008). In other words, dioecy is probably stable to the invasion of hermaphrodites because of the advantages of gender specialisation. We find it hard to conceive of conditions under which hermaphrodites with intermediate sex allocation would be disfavoured by selection under the same conditions that would favour slightly leaky males and females.

Whereas inconstant sex expression might be disadvantageous in dense populations of *M. annua*, it might confer an advantage in sparse populations where mates are limiting (Ehlers and Bataillon, 2007; Crossman and Charlesworth, 2014). Such conditions may occur frequently in *M. annua*, which colonizes disturbed habitats (Pannell 1997d, 2000). The ability to self-fertilize would be advantageous for both males and females during critical phases of colony establishment (Baker 1967; Pannell and Barrett 1998; Pannell *et al.* 2015). It is thus plausible that, in species like *M. annua* in which mate availability may vary, dioecy with leaky sex expression might be selected, even if leaky dioecy were disadvantageous in populations with abundant mates. Such a strategy would be particularly advantageous for individuals that can respond plastically to a paucity of mates by producing flowers of the opposite sex (Charnov *et al.* 1976).

Gender switching in response to density (and thus to mating opportunities) has previously been reported for androdioecious *M. annua* in hexaploid populations (Pannell 1997d; though see Sánchez-Vilas and Pannell 2012), and we have noticed a somewhat greater tendency for females of dioecious *M. annua* to produce male flowers when they are highly pollen-limited (e.g., to show leaky sex expression; J.R. Pannell, personal observation). Such mating-context-dependent gender choice does not appear to be common, but it is well described for homosporous ferns (Haig and Westoby 1988; Tanurdzic 2004). In some homosporous ferns, spores develop as pure males in the presence of chemical signals produced by nearby hermaphrodites whose eggs they might fertilize, but as self-fertile hermaphrodites when potential mates are absent (Warne and Lloyd 1987; Korpelainen 1998). It is not known how widely such plasticity is found in angiosperms, but it would seem to be a trait that plants should evolve if they could assess their mating opportunities accurately (Charnov *et al.* 1976).

Selection of males and/or females of *M. annua* with leaky sex expression during episodes of colonisation could also explain the asymmetry of inconstancy observed, both in terms of its rate and its degree, because a selfing ability should have unequal benefits for males versus females (Pannell, 2000). While the production of small amounts of pollen by an inconstant female may be sufficient for the fertilization of a large number of her seeds, allowing the quick establishment of a population of viable size, not least if inbreeding depression is low, as in dioecious *M. annua* (Eppley and Pannell 2009). In contrast, the few seeds produced by inconstant males would establish a much smaller population, which is more likely to become extinct through demographic stochasticity (Pannell *et al.* 2015). We might thus expect a higher rate of sex inconstancy to be found among females than among males. When sex inconstancy in males is advantageous in colonizing

situations, selection should favor a high *degree* of male inconstancy, not only by enhancing colonization success, but also because selfing favors increased allocation to female functions (Charlesworth and Charlesworth 1981; Charnov 1987; De Jong *et al.* 1999).

Our results throw new light on the evolution of androdioecy in the *M. annua* species complex. Although the populations studied here are functionally dioecious, hexaploid populations of *M. annua* are frequently androdioecious, with males co-occurring with monoecious hermaphrodites. Previous research suggested that androdioecy in hexaploid *M. annua* might have evolved through the introgression of a Y chromosome (and thus males) into monoecious populations through allopolyploid hybridization (Obbard *et al.* 2006), i.e., that androdioecy have evolved under the unusual circumstances of Y-chromosome introgression into a polyploid hermaphroditic background. While our current results do not reject this hypothesis, they suggest that even diploid dioecious populations of *M. annua* are prone to the evolution of androdioecy because, unusually, they have a greater frequency of inconstant females than males. Alternatively, female inconstancy in *M. annua* could reflect a past history of androdioecy.

Underlying physiological basis of inconstancy in *M. annua*

Whatever its external cause, inconstant sex expression in *M. annua* is likely mediated by altered hormone signalling within the plant. Plant hormones regulate many aspects of plant growth, including responses to environmental stressors (Voesenek and Blom 1996; Sultan 2000; Chapman and Estelle 2009), and they play a key role in flowering, including its timing (Barth et al. 2006; Barazesh and Mcsteen 2008; Song et al. 2013; reviewed in Golenberg and West 2013) and the relative position of staminate versus pistillate flowers in monoecious individuals (Rood and Pharis 1980; Freeman *et al.* 1981). In *M. annua*, the balance between cytokinins and auxins determines whether floral primordia develop as male or female (Durand 1963; Dauphin-Guerin *et al.* 1980; Louis *et al.* 1990; Durand and Durand 1992), and exogenous application to males of the feminizing hormone benzylaminopurine (6-BAP) causes them to produce fully functional pistillate flowers (Louis and Durand 1978; Durand and Durand 1991). Sex inconstancy in dioecious *M. annua* may therefore reflect variation either in hormone levels within the plant and/or of variation in tissue sensitivity to hormones during development, mediated by environmental variation.

Phytohormones affect the expression of multiple traits, and their concentration may be modulated by various environmental factors (e.g., Broekaert et al. 2006; Kazan and Manners 2009). Labile sex expression might thus plausibly reflect responses to environmental variation (Korpelainen 1998;

Golenberg and West 2013). To the extent that such lability has a heritable component, hormone-mediated expression of sex inconstancy could expose phenotypic variation to selection through genetic assimilation (Waddington 1953; Crispo 2007; Lande 2009). Because of the modularity of growth and reproduction and the role played by hormones in regulating the production of male versus female flowers, monoecious plants may be particularly susceptible to frequent shifts between combined versus separate sexes, and may be suitable as models for studying genetic assimilation as an important factor in allowing transitions between contrasting phenotypic strategies.

Broader implications of sex inconstancy in flowering plants

While unusual, *M. annua* is not the only species showing greater inconstancy in females than males For instance, Freeman and McArthur (1984) studied sex allocation in a number of *Atriplex* species that display occasional complete sex reversal. In *Atriplex canescens, they found that females* were more frequently inconstant than males. Similarly Huff and Wu (1992) found a slightly higher frequency of inconstant females than inconstant males in the grass *Buchloe dactyloides*. Both of these species bear unisexual flowers, and probably had monoecious ancestors. Although dioecy is ancestral in the genus *Mercurialis* (Obbard *et al.* 2006), monoecy is very common in the family Euphorbiaceae in which bisexual flowers are not known. Thus it is likely that dioecy in each of these species with greater female inconstancy evolved via monoecy, too.

The selection of inconstancy in dioecious colonising species should also depend on the genetic mode of sex determination (Lloyd 1975a). If males are the heterogametic sex, such as *M. annua*, populations colonized by inconstant XX females could establish self-sustaining populations of inconstant females homozygous for the factor leading to females (analogous to the X chromosomes in an XY sex-chromosome system). Competition among these females to sire seeds should select for increased allocation to male function, establishing populations of hermaphrodites that all produce substantial amounts of pollen (Dorken and Pannell 2009). Species that have evolved dioecy through the gynodioecy pathway tend to show only male inconstancy so that the breakdown can occur solely through selection on male inconstancy. However when considering the paradioecy pathway, selection on initially inconstant females under mate limitation could thus have been an important step in the breakdown of dioecy and the evolution of hermaphroditism (Pannell 2000; Wolf and Takebayashi 2004; Delph 2009), as well as in certain animals (Pannell 2008). In contrast, populations founded by inconstant heterogametic males will immediately segregate both sons and daughters, re-establishing a dioecious population. The breakdown of dioecy in species with male hetergamety should thus perhaps rarely involve selection on inconstant males. Similarly, in species

with female heterogamety, populations founded by inconstant females will immediately segregate both sons and daughters, re-establishing a dioecious population. Indeed, such a process was invoked by Lloyd (1975a) for transitions to monoecy in *Leptinella* (*Cotula*), a species with female heterogamety.

Concluding remarks

We have adopted Lloyd's quantitative approach to gender and sex allocation (1980b) to characterise leaky sex expression in *M. annua*; this approach has been widely used to assess functional gender of plants in different dioecious and subdioecious species (e.g., Campbell 1989; Delph and Lloyd 1991; Verdu *et al.* 2004). Our approach emphasises the importance of considering separately two aspects of inconstancy, its rate and its degree. We believe that this may be particularly valuable when considering inconstancy in a context of sexual-system transitions. The degree of inconstancy informs us directly about the extent to which a particular sex may depart from its 'pure' form, relatively of the 'pure' form of the opposite gender, so that it permits us to account for the functional significance of inconstancy in the population. The rate of inconstancy may be particularly important for informing us on the likelihood of sexual-system transitions occurring through modification of one sex versus the other, and to formulate expectations on the potential direction of a breakdown of dioecy via selection of sex inconstancy.

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FIGURES

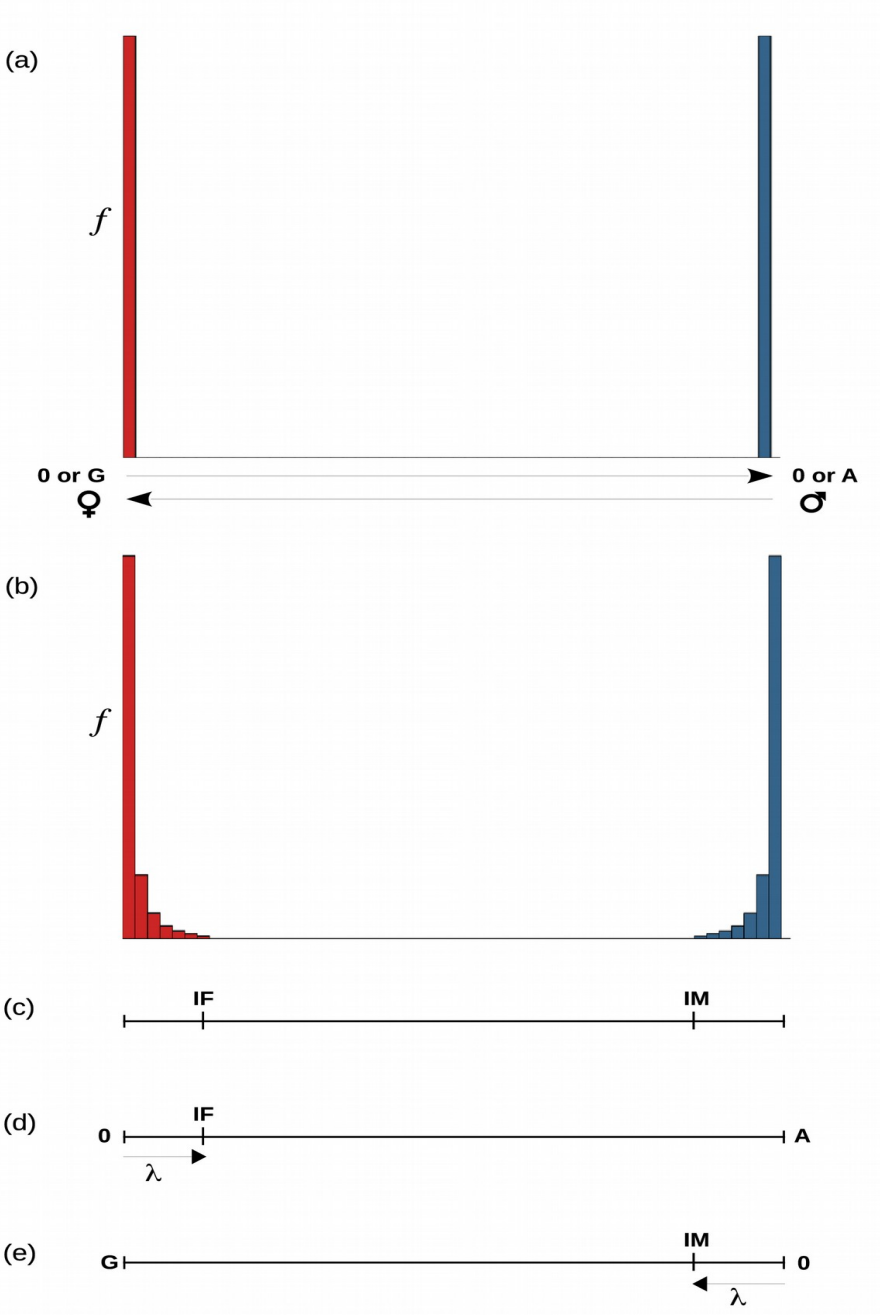

Figure 1. Cartoon representing a continuum between 'pure' female (left-hand side in red) and male allocation (right-hand side, in blue). **(a)** Density barplot of sex allocation in the case of a perfectly canalized population with no inconstancy, i.e., where males and females invest all their reproductive resources into their main sexual function. The reproductive effort of constant females and males is *G* and *A*, respectively. **(b)** Scenario in which a fraction of individuals invest a part of their biomass into opposite sex function. **c)** Depiction of the continuum between 'pure' males and 'pure' females along which inconstant females (IF) and inconstant males (IM) may be located. **(d)** and **(e)** present measures of λ_f and λ_m on the sex-allocation continuum.

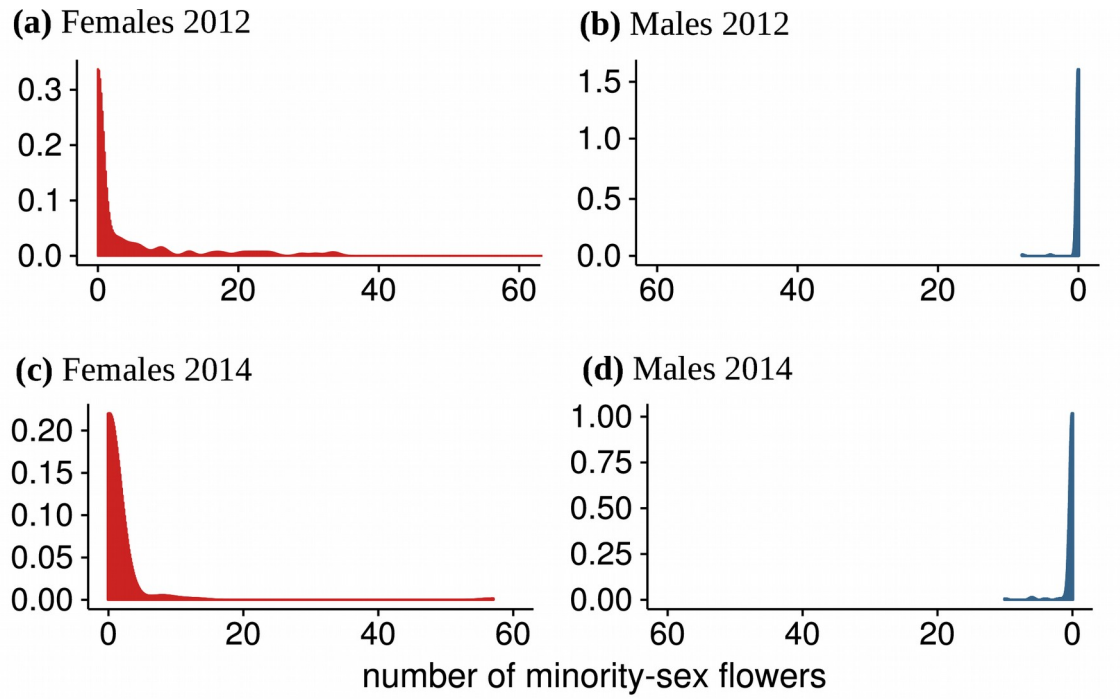

Figure 2. Density plot of the number of minority-sex flowers produced by females (red) and males (blue) of *M. annua*. The top row shows data from 2012 and the bottom row shows data from 2014.

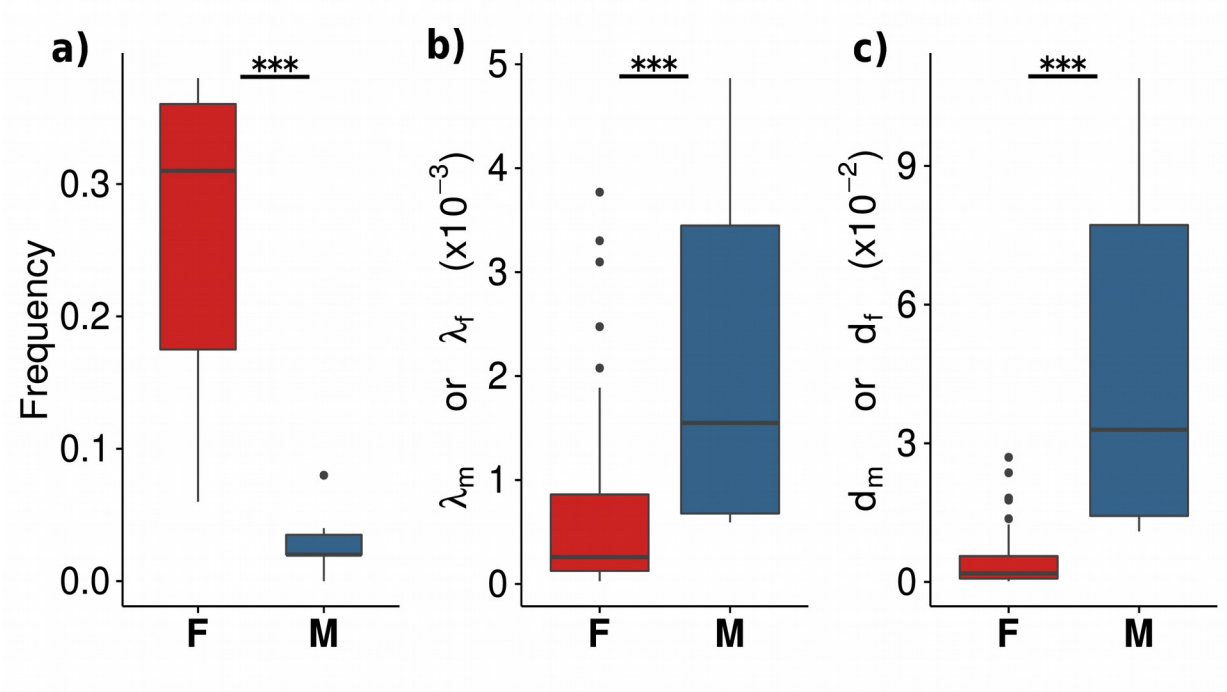

Figure 3. Boxplots of the descriptive statistics of inconstancy averaged across the three experimental populations of *M. annua*, for 2012 and 2014 together. **(a)** Rate of inconstancy. **(b)** Inconstant reproductive effort, λ_f and λ_m. **(c)** Normalized inconstant reproductive effort, *d*. Significant differences revealed by Fisher or Wilcoxon tests are indicated with asterisks. Data for females and males are shown in red and blue, respectively.

TABLES

Table 1. Means and standard deviations on measured phenotypic traits, averaged per population, measured on **(a)** canalized individuals, **(b)** inconstant individuals (except for d_{all}, see Materials and Methods), and **(c)** inconstancy indexes. Fisher exact tests have been used to compare rates of inconstancy; Wilcoxon tests (two-sided) have been used for biomass, *d*, and *r.*

a) Canalized individuals

Variable	2012			2014		
	Males	**Females**	**P**	**Males**	**Females**	**P**
Sample size	49.7 +/- 1.15	31.3 +/- 0.58	-	47.3 +/- 1.15	42.00 +/- 5.57	-
Biomass	3.14 +/- 0.98	4.34 +/- 2.29	2.3×10^{-2} *	3.95 +/- 1.25	5.28 +/- 1.22	5.83×10^{-8} *
Number of staminate flowers	NA	0	-	1822 +/- 288	0	-
Number of pistillate flowers	0	157 +/- 74.1	-	0	109 +/- 16.7	-
Biomass of staminate flowers (g)	-	0	-	0.55 +/- 0.09	0	-
Biomass of pistillate flowers (g)	0	0.32 +/- 0.17	-	0	0.23 +/- 0.04	-
Nitrogen of staminate flowers (µg)	NA	0	-	24.91 +/- 3.94	0	-
Nitrogen of pistillate flowers (µg)	0	5.99 +/- 3.24	-	0	4.30 +/- 0.81	-

b) Inconstant individuals

Variable	2012			2014		
	Males	**Females**	**P**	**Males**	**Females**	**P**
Biomass	2.81 +/- 0.47	5.42 +/- 3.10	0.254	5.48 +/- 2.75	6.21 +/- 1.64	0.104
Number of staminate flowers	NA	12.7 +/- 8.87	-	1928 +/- 78.6	5.52 +/- 5.58	-
Number of pistillate flowers	6.00 +/- 2.83	201.6 +/- 90.0	-	4.25 +/- 2.41	123.7 +/- 15.9	-

Biomass of staminate flowers (g)	NA	3.86×10^{-3} +/- 2.7×10^{-3}	-	0.59 +/- 0.02	1.68×10^{-3} +/- 1.7×10^{-3}	-
Biomass of pistillate flowers (g)	1.82×10^{-3} +/- 8.59×10^{-4}	0.39 +/- 0.21	-	1×10^{-2} +/- 1×10^{-2}	0.27 +/- 0.07	-
Nitrogen of staminate flowers (µg)	NA	0.17 +/- 0.12	-	26.4 +/- 1.07	0.075 +/- 0.076	-
Nitrogen of pistillate flowers (µg)	0.035 +/- 0.016	7.39 +/- 3.99	-	0.17 +/- 0.096	5.15 +/- 1.30	-

c) Inconstancy indexes

Variable	**2012**			**2014**		
	Males	**Females**	**P**	**Males**	**Females**	**P**
r	0.013 +/- 0.012	0.363 +/- 0.020	$<2.2 \times 10^{-16}$ *	0.0467 +/- 0.031	0.16 +/- 0.11	1.97×10^{-3} *
A_n	NA	-	-	476 +/- 80.2	-	-
G_n	-	36.8 +/- 2.16	-	-	21.2 +/- 3.76	-
A_b	NA	-	-	0.14 +/- 0.024	-	-
G_b	-	0.073 +/- 9.9×10^{-4}	-	-	0.044 +/- 0.01	-
λ	6.83×10^{-4} +/- 4.2×10^{-4}	7.80×10^{-4} +/- 1.66×10^{-4}	0.62	1.69×10^{-3} +/- 1.09×10^{-3}	3.25×10^{-4} +/- 3.01×10^{-4}	1.00×10^{-4} *
d_{inc}	9.37×10^{-3} +/- 5.7×10^{-3}	5.39×10^{-3} +/- 5.9×10^{-3}	0.20	4.63×10^{-2} +/- 3.7×10^{-2}	2.27×10^{-3} +/- 5.9×10^{-3}	5.32×10^{-6} *
d_{all}	1.33×10^{-4} +/- 1.21×10^{-3}	2.08×10^{-3} +/- 4.53×10^{-3}	6.22×10^{-14} *	2.18×10^{-3} +/- 1.24×10^{-2}	3.68×10^{-4} +/- 1.80×10^{-3}	2.47×10^{-3} *

* : significant at $P < 0.05$.

Table 2. Summary statistics of the hurdle model. Only the final model is presented with significant variables. We modelled variation in terms of a mixed generalized linear model, with the number of flowers of the opposite sex a function of the year and sex (fixed factors) assuming a negative binomial error distribution, and using a binomial distribution to model the probability to measure zero flower of the opposite sex.

Count model (negative binomial)	Estimate	Std. Error	*P-value*
Intercept	1.753	0.554	$1.28\ x10^{-3}$
Year	- 1.107	0.400	$5.65\ x10^{-3}$
Log(theta)	- 1.694	0.757	$2.52\ x10^{-2}$
Zero hurdle model (binomial with logit link)			
Intercept	- 0.554	0.171	$1.17\ x10^{-3}$
SexM	- 3.757	0.732	$2.87\ x10^{-7}$
Year	1.104	0.281	$8.37\ x10^{-5}$
Sex:Year	2.405	0.858	$5.04\ x10^{-3}$

- CHAPTER 4 -

Selection experiment on inconstant male allocation by females of *Mercurialis annua* by manipulation of population sex-ratios

Guillaume Cossard and John R. Pannell

Introduction

Flowering plants display a great diversity of sexual systems, which range from hermaphroditism, where both sexes are combined into the same flower, to dioecy, where sexual functions are separated between different individuals (Darwin, 1877; Renner & Ricklefs, 1995; Barrett, 2002). About 90% of angiosperm species are hermaphroditic, while only a minority (~ 6%) have fully separate sexes (Renner, 2014). The rarity of dioecy has long been viewed as a consequence of a lower diversification rate of dioecious clades, characterized as evolutionary dead-ends (Heilbuth, 2000). However a number of recent studies (Käfer *et al.*, 2014, 2017; Sabath *et al.*, 2016) have demonstrated that these clades likely diversify at the same rates as hermaphroditic ones. Hence, the loss of dioecy is perhaps less likely to be explained by excessive extinction events than by the possibility of reversion to hermaphroditism (Käfer *et al.*, 2017). Indeed recent studies have shown that hermaphroditism may often be derived from dioecious ancestors (Liston *et al.*, 1990; Obbard *et al.*, 2006a; Schaefer & Renner, 2010; Barrett, 2013; Käfer *et al.*, 2014; Renner, 2014).

In a recent review, Kafer *et al*. (2017) suggested that three mechanisms could in fact lead to a shift away from dioecy. First, if separate sexes have evolved as a mechanism to avoid selfing and inbreeding depression, any process leading to the erosion of inbreeding depression (e.g., purging of genetic load, loss of genetic diversity after colonization) may allow self-fertile hermaphrodites to re-invade and spread in a dioecious population (Charlesworth & Charlesworth, 1978; Lande & Schemske, 1985; Pannell *et al.*, 2015). Second, a change in the fitness set, i.e., the trade-off between male and female allocation, may be advantageous for hermaphroditic strategies in cases where increasing allocation to one gender or the other translate into decreasing marginal fitness gains (Charnov *et al.*, 1976; Charnov, 1982). Finally, self-fertile hermaphroditism may be beneficial when mates, or pollinators, are scarce, as it provides a guarantee for reproduction by selfing (Baker, 1955, 1967; Pannell, 2015; Pannell *et al.*, 2015) while it conserves the possibility to outcross, too, providing a transmission advantage over unisexual individuals that can only outcross pollen (Fisher, 1941). Such mate limitation may often be experienced by colonizing species, e.g., species that form metapopulations constituted of demes with high population turnover rates, or in species with density-dependent selfing rates. Thus, ecological mechanisms affecting the mating environment are potential causes of sexual-system shifts (Ashman, 2006; Dorken & Pannell, 2009), as supported by population genetic models (Ehlers & Bataillon, 2007; Crossman & Charlesworth, 2014).

Ecological variation occurs over short time scales and can lead to rapid evolutionary changes, eventually leading to speciation. Classical animal examples of rapid evolutionary changes include the radiation of Darwin's finches (Grant & Grant, 2006; Almén *et al.*, 2016), that of cichlid fishes in

African lakes (Seehausen, 2006) or the evolution of fur colour from ancestral variation in deer mice (Steiner *et al.*, 2007; Domingues *et al.*, 2012). Rapid evolution on ecological time scales has also been reported for plant populations, e.g., involving heavy metal tolerance (Wu *et al.*, 1975), adaptation to fertilizer treatment (Silvertown *et al.*, 2006), or adaptation to new environments in the case of introduced species (Sultan *et al.*, 2013; Vandepitte *et al.*, 2014). These rapid evolutionary changes imply adaptation in response to selection on standing heritable trait variation in the population (Barrett & Schluter, 2007).

Reproductive traits are often found to be polymorphic in plant populations (Gigord *et al.*, 2001; Barrett, 2002), and a number of studies have recorded fast adaptive changes of reproductive traits in response to natural (e.g., Campbell, 1989; Dorken & Pannell, 2009; Hopkins & Rausher, 2012) or artificial selection (e.g., Lendvai & Levin, 2003; Delph et al., 2004a,b; Wright et al., 2005). Polymorphic sex allocation is typical of natural plant populations (Darwin, 1877; Stebbins, 1950), so that it is common to observe ‘leakiness’ in sex expression, or ‘inconstancy’, in dioecious populations (e.g., Lloyd, 1972a; Diggle, 1991; Korpelainen, 1998; Delph, 2003; Venkatasamy *et al.*, 2007), i.e., the ability for some individuals to occasionally produce functional gametes of the opposite sex. Plant breeders have long noted the presence of inconstancy in dioecious species and artificially selected on this character to produce functionally hermaphroditic strained derived from dioecious lineages, e.g., in the many monoecious cultivars of *Cannabis sativa* (Moliterni *et al.*, 2004), or in *Actinidia deliciosa* (Mcneilage & Steinhagen, 1998; Ferguson & Huang, 2007).

Inconstancy may originate from occasional recombination in the sex-determining region, allelic variation at loci involved in sex allocation, uncanalised phenotypic expression and developmental instability, and/or plasticity. Species that have recently evolved dioecy may experience imperfect segregation of sex-determining factors, resulting in the co-expression of both genders (Crossman & Charlesworth, 2014) or in the production of neuter individuals that are sterile for both functions (Spigler & Ashman, 2011). Alternatively, plasticity is an important factor that brings about inconstancy in sex allocation in dioecious plant populations, i.e., where inconstancy is more than just unstable expression of the genotype but an evolved sex-allocation reaction norm (Korpelainen, 1998; Sultan, 2000; Delph & Wolf, 2005). For example, species with a metapopulation structure and distributed over variable environments might benefit from such plastic strategies, e.g., if their phenotype were sensitive to whether mates were present or not (Sultan & Spencer, 2002). Of course, in addition to bringing about the conditions that would favour hermaphroditism, the biotic and abiotic environment may have a direct influence on the sex expression of individuals, directly

bringing about inconstancy in sex expression (Charnov, 1982; Delph, 2003; Dorken & Mitchard, 2008; West, 2009; Golenberg & West, 2013).

Inconstancy has previously been hypothesized to be the starting point of evolutionary shifts away from separate sexes (Pannell, 2000; Golenberg & West, 2013; Käfer *et al.*, 2017). Lloyd (1972b, 1975a,b), in a seminal study on New Zealand populations of several species of *Leptinella*, referred to variation in sex allocation among species in speculating about how monoecious populations can be derived from dioecious ones through the spread of inconstant males in the response to selection for uniparental reproduction due to mate limitation. The possibility of such reversions from dioecy strongly depends on the patterns of inconstancy that we observe in a plant population. As discussed in Chapter 3, two general patterns of inconstancy occur in natural dioecious populations: (i) inconstancy in males only; or (ii) inconstancy in both sexes. These patterns probably reflect the evolutionary history of the separation of sexes in a particular lineage (Lloyd, 1980). The former is thought to be the result of the evolution of dioecy through the 'gynodioecy pathway', where females are determined by a major recessive (loss-of-function) sterility mutation that, when in homozygotes, prevents them from inconstant sex expression (Charlesworth & Charlesworth, 1978), whereas males, which have gradually evolved decreased female functions, may often retain some (Lloyd, 1980). In contrast, the expression of leakiness in both sexes is more likely to reflect a monoecious ancestral state from which males and females have departed under the effect of disruptive selection for canalized sex allocation at the two gender extremes (Charnov, 1982).

In chapter 3, we demonstrated that both gender were inconstant in *M. annua* although they usually differ in the pattern of inconstant sex expression, with females being more frequently inconstant than males. We found that, in the presence of specialized males, the functional significance of female inconstancy was low, as reflected by the low normalized inconstant reproductive effort (d_{all}), we measured. We hypothesized that males may adaptively maintain inconstancy at a low level in females but that transient episodes of isolation from males, during isolation or colonization, may occasionally benefit enough inconstant females to explain the maintenance of labile sex expression in dioecious populations. Indeed the functional gender of inconstant females would drastically change in situation of isolation, because the low amount of pollen they produce would sire a more significant fraction of the seeds in the mate-limited or isolated population. *M. annua* is a colonizing species that may often experience isolation after seed dispersion (Pannell, 1997), potentially favouring inconstant phenotypes (Pannell, 2000), if this trait is under the control of genetic variation.

Inconstant sex expression is likely to be underlined by heritable genetic variation, as has been found in *Buchloe dactyloides* (Huff & Wu, 1992). Inconstancy could be mostly encoded by a major gene (or quantitative trait locus, QTL) underlying much of the genetic variance, as is the case of several traits related to floral morphology in *Mimulus lewisi* and *Mimulus cardinalis*, for instance (Bradshaw *et al.*, 1998). Conversely inconstancy may be underlined by many loci (or QTL) of small effects throughout the genome, as in the case of flowering time in maize (Buckler *et al.*, 2009). The genetic architecture of inconstant sex expression is likely to influence the response to selection and the pace of evolutionary shifts when isolated from males, as well as the distribution of trait variation during the course of evolution. Alternatively, if inconstancy is solely the outcome of plasticity and does not reflect any associated genetic variation, we should not of course expect to observe any change in phenotypes distribution. Ultimately, the response to selection relates to the heritability of this trait, h^2, measured as the ratio of additive genetic variance, V_A, over the total phenotypic variance observed in a population (V_P), i.e., $h^2 = V_A / V_P$ (Lynch & Walsh, 1998).

In the present study, we conducted experimental evolution on dioecious populations of the annual herb *Mercurialis annua* to test whether isolation from males can select on inconstancy *via* reproductive assurance and benefit from outcrossing, and thus bring about a shift in the sexual system of these populations. Natural dioecious populations of *M. annua* display 1:1 sex ratios and are characterized by sexual dimorphism, with females typically investing more resources in above-ground tissues while males tend to be smaller and lighter (Harris & Pannell, 2008; Sánchez-Vilas & Pannell, 2011; Sánchez-Vilas *et al.*, 2011). Moreover females produce sessile flowers at the leaf axils, which contrasts with male inflorescences erected on peduncles, probably as an adaptive specialization for pollen dispersion. In the present experiment, we simulated mate limitation and isolation by removing males from large experimental populations. Although this design is biologically implausible in its detail, i.e., we do not expect to observe large natural populations of *M. annua* constituted only of females, this design (Figure 1) bears the double advantage of recreating the conditions under which we expect inconstancy to be positively selected (low male frequency), while potentially maintaining reasonable amount of genetic variation, lowering the possible effects of genetic drift. In one sense, our experiment recreates situations where many isolated females across a potential metapopulation are simply placed close together for logistical reasons (though see Discussion). Our experiment allowed us to address the following points: (i) Are polymorphic sex allocation patterns of dioecious *M. annua* underlined by heritable genetic variance? (ii) Can a modification of sex ratio in a population be susceptible to bring about a shift of sex allocation in populations? (iii) To what extent should isolated females respond to selection and evolve towards a functionally hermaphroditic strategy?

Materials and methods

Experimental design

We established our replicated control and selected lines based on a common seed set from a pool of 36 populations of dioecious *M. annua* across Europe and East Mediterranean (Turkey, Israël), used previously (Labouche & Pannell, 2016; Chapter 3), i.e., we sampled from the Europe-wide 'metapopulation' of *M. annua*. In the late spring 2012, for each replicate we sowed 300 seeds in cell trays filled with a mixture of horticultural soil and perlite. We let them grow under controlled greenhouse condition until early flowering, allowing us to discriminate between males and females. Sex ratios of seedlings were recorded at this point. We set six artificial replicates of 210 individuals of *M. annua*, divided between two treatments: (i) three populations consisting only of females (selected lines); and (ii) three populations with 1:1 sex ratio (control lines). The very low seed set of males lines did not permit us to set male-only lines that would produce enough offspring to establish male-only lines for the continuation of the experiment. Females were randomly assigned to these replicates and males to control lines only (Figure 1). Replicates were set on the campus of the University of Lausanne and in private gardens around Lausanne, in order to maintain a sufficient distance to avoid cross pollination among plots (Eppley & Pannell, 2007). The geographic location of each replicate was randomized for each of the three first generations, and each location was checked to remove possible naturally occurring *M. annua.* From generation 4, we maintained populations at the same locations each year to avoid potential gene flow from control to treatment plots via plants that might have established in the habitat from previous generations; we nevertheless continued to survey sites carefully and to remove any stray individuals, and indeed we found no evidence of contamination during analysis (see Results). This Chapter reports results from selection over the first four generations, but the experiment is continuing. In the summer 2016, after the fourth generation of selection was completed, we grew 30 females from each replicate of each generation, as well as 90 females from the initial seed pool (generation 0), along with males from control lines, in a common garden at the University of Lausanne, to estimate the response to selection. The plant density was kept high in the common garden, so that we expect females were not pollen-limited under these conditions.

Data recorded

During the experiment, individuals of each replicate were allowed to mate freely in experimental sites for approximately seven weeks. At harvesting time, we recorded the following traits on 50 randomly chosen females per replicate: the number of male flowers (before anther dehiscence) and seeds produced, the above-ground height and drymass, as well as seed weight. Seeds from each replicate were used to set the next generation of the same line the following year by subsampling

from the seed progeny of the previous year. Seeds not used were stored in air-tight containers at 4° C for later use (see below). Records of the seedling sex ratios permitted us to estimate the level of possible pollen contamination from any naturally occurring *M. annua* males around our experimental populations that might have been missed; given that sex is determined by male heterogamety in *M. annua* (Russell & Pannell, 2015), pollen flow from surrounding male pollen in our female-only populations would result in the production of males in the progeny of our experimental lines (see Results).

For each individual of the common garden, we recorded the same traits as in the field replicates, as well as weight of male flowers produced by males. Measures from the field were taken from adult plants undergoing selection at a given generation, while measures from the common garden were taken from germinated seeds produced at the corresponding generation (Figure 1).

For each recorded plant, both from field replicates and common-garden data, we computed male reproductive effort (MRE) as the ratio of male flower weight over the above-ground biomass. This corresponds to our measure of inconstancy, λ, used in Chapter 3. We calibrated the average mass of a single male flower from 35 females from the common garden in order to compute the total weight of male flowers when it was too low to be measured by our balance (which was precise to 0.00001 g). Similarly, we computed female reproductive effort (FRE) as the total matured seed weight over above-ground biomass, and an index *V*, as the ratio of height over biomass. We computed the degree of inconstancy, *d*, of females as the ratio $MRE_{females}$ / MRE_{males}, calculated across females of each replicated line separately, relatively to males in control lines from the same generation (see Chapter 3).

Statistical analysis of the response to selection

For the field replicates, we calculated females' relative fitness following Morgan & Schoen, (1997). Following Dorken & Pannell (2009), we estimated directional selection (β) and nonlinear selection gradients (γ) as the linear and quadratic regression coefficients of MRE on relative fitness (Lande & Arnold, 1983). Quadratic selection gradients were calculated as twice the quadratic regression coefficient (Lande & Arnold, 1983; Stinchcombe *et al.*, 2008). We used Mann-Whitney ranked tests to compare MRE, FRE and above-ground biomass of females between selected and control lines, at each generation, in both field replicates and common garden. The phenotypic response to selection was calculated from females in the common garden as the mean difference of MRE between generation four and the base population (Δ_{MRE}), and as the number of MRE standard deviation present in the initial generation (Δ_{sd}).

We calculated the selection differential *S* as the covariance between MRE and relative fitness independently for each of the field populations undergoing selection. Using the breeder's equation (Lynch & Walsh, 1998), we calculated the narrow-sense heritability as $h^2 = \Delta_{MRE} / S$, averaged across the three selected lines after the three last rounds of selection. Values for the response to selection (Δ_{MRE}) used in the calculation of h^2 were taken from field populations (to match the selection differential estimates). We averaged across the three years to obtain an estimation of h^2 of female MRE.

Results

Pollen limitation in selected lines

From the first generation, female-only lines produced enough pollen to sire seeds and to permit to establish the following generation. After the first generation of selection, we recorded the presence of a few males in the progeny of each of the three selected lines (1.33% of germinated seedlings in total), which likely points to a low level of pollen contamination from surrounding naturally occurring males of *M. annua*. Mean FRE was lower in selected lines compared to control lines (Figure 2; Table 1), especially during the first generation, probably indicating pollen limitation in experimental lines during this generation. From the third generation on, females from the selected lines produced only females in their progeny, while the difference in FRE between control and selected lines was reduced, albeit significant again at generation 3 (Figure 2; Table 1).

Male reproductive effort variation in females of base populations

The initial seed pool used to constitute both control and selected lines, i.e., generation 0 in the common garden, corresponds to the plants growing at generation 1 in the field populations, i.e., undergoing the first round of selection. There was substantial variation in male sex allocation among females of this base population in the field (Table 1, Figure 3c, Figure 4). The mean MRE of females in the base population was 1.58×10^{-4} ($SD = 5.44 \times 10^{-4}$, Table 2) and the mean degree of inconstancy observed (0.90, $SD = 3.08$) was slightly inferior to what we observed in Chapter 3. There was substantial variance of MRE within the base population and we recorded a high standard deviation value of MRE in the common garden ($SD = 5.44 \times 10^{-4}$). We measured the frequency of females above this threshold in control and selected lines. In the common garden, we recorded on average 47.5 % of females above this threshold among females of the base population (Figure 3c). In the field, male reproductive effort of females in selected lines was 2.32 times that of females in control lines during the first round of selection (Figure 4a).

Response to selection in experimental lines and heritability

We measured significant positive directional selection gradient acting on male reproductive effort in the selected lines at each generation, on average across replicates (Table 3). The response to selection was inferred from the common-garden data. Selected lines responded quickly and positively to the selection regime imposed, with a significant increase in the MRE of females after the first round of selection. Fold change in MRE in selected females compared to control lines increased from 8.77 after the first round of selection to 23.3 after four generations of selection, on average across replicates (Table 2, Figure 3b). This represents a response to selection, Δ_{sd}, of 5.84 times the standard deviation of MRE in base population, after four generations of selection (Figure 5). The frequency of inconstant females allocating more to male function than the mean level at generation 0 (1.58×10^{-4}) in selected lines consistently increased during our experiment, from 0.85 after the first generation of selection, to 0.95 after the fourth generation, averaged across experimental populations.

Using the measure of selection differential from the selected lines and their observed response to selection, we estimated the narrow-sense heritability for each generation of selection. We found h^2 to be equal to 0.66, 0.28 and 1.24 at generations 1, 2 and 3, respectively. We estimated the mean heritability of this trait across years, to be 0.73 (SD = 0.48). Note that our calculation of h^2 was superior to 1 at the last generation,which is clearly an artefact of the way it was calculated. I address this point in the Discussion.

In the selected experimental populations, a minor fraction of females remained ‘canalized’ (no production of male flowers), while others produced a large number of male flowers, except for one experimental line that contained only females with high MRE (Figure 3c). These modified females carried clusters of male flowers beside female flowers in the leaf axils, including those towards the apex of the plant (Figure 3a). None of these individuals displayed pedunculate inflorescences typical of males of *M. annua*.

Phenotypic distribution of male reproductive effort in the selected lines

There was both significant directional selection on the MRE of females in the selected lines (Table 3), whereas we detected no significant gradient of selection in the control lines. We detected slightly significant quadratic gradients in selected lines at the first and third round of selection. They were, however, monotonic and thus give no indication of disruptive selection. The variance for MRE within experimental populations increased in the selected lines during the experiment, but not in the

control lines (Figure 6). In the common garden, variance in MRE was 2.96 $x10^{-7}$ (SE = 3.24 $x10^{-6}$) in the base population, and became 1.13$x10^{-7}$ (SE = 5.45 $x10^{-8}$) and 2.05 $x10^{-5,}$ (SE = 5.45 $x10^{-8}$), on average, in control lines and selected lines, respectively (Figure 6). This increased variance is driven by the fact that some females of the selected lines invested heavily in male functions while others remained canalized and produced only seeds, like most of the females in the control lines. Females with high MRE were observed from the first generation of selection in selected lines (Figure 6), and the highest level of MRE we measured during the course of our experiment occurred in a selected line after the third round of selection (Figure 6, green curves). No females in the following generation displayed such male-biased allocation. The distribution of MRE among females of our experimental populations differed substantially between control and selected lines (Figures 6 and 7). Control lines showed a skewed distribution, with many females with a null MRE, maintained across the four generations of selection. Selected lines responded strongly to selection from the first generation of selection: in the first two generations, most of the females displayed a similar slight increase in MRE, in the two last generations of selection MRE of male-deprived females varied significantly among them, with no particular value of MRE more frequently observable (Figure 7).

Change in life-history traits associated with shift of MRE

Along with the increase of MRE in selected females, we recorded several related life-history traits known to vary with male sex allocation: height, above-ground biomass, and female reproductive effort. After the first round of selection, females of selected lines displayed a significantly lower height/biomass ratio in the common garden (P = 6.86 $x10^{-4}$; Table 2), and a higher female reproductive effort (P = 8.15 $x10^{-3}$; Table 2). Differences in V between females of selected and control lines decreased at generation 2 and were not detectable in the two last generations. In the meantime, control-line females tended to increase their FRE, so that no significant difference was detected at generations 2 and 3, while females from control lines invested significantly more in female functions than did females from the selected lines after the fourth generation (P = 0.022; Table 2; Figure 8).

Discussion

This study set out to examine the potential response to selection on leaky male expression of females of dioecious *M. annua* when males are removed from their populations. The results were dramatic: females evolved an ever-increasing allocation of resources to their male function over the course of only four generations, with their mean male reproductive effort being, on average, 21-fold that of females in the base population. The response we observed was as soon as the first round of selection occurred, and was reflected in both the increased frequency of pollen-producing females,

as well as in the extent to which these modified females invested in male functions. While it is clear from our results that much of the variation we observed in leaky sex expression and male reproductive effort can be attributed to plasticity, the response to selection indicates that variation in these traits has a clear genetic component, and that trait values are strongly heritable. Below, we discuss the relative importance of plasticity and genetic variation in female inconstancy revealed by our study, beginning with a consideration of the implications of the broad genetic base from which we had sampled *M. annua* for establishing our replicate populations. We then consider the implications of our results for our understanding of both the remarkable variation in sexual expression displayed by the *M. annua* species complex as well as ideas about transitions between combined and separate sexes in plants more generally.

Variation in leakiness in the base population

In order to establish our experimental evolution study, we collected seeds from across the distribution range of dioecious *M. annua*. This sampling design allowed selection to act on variation across the species range, i.e., across the global metapopulation, rather than on variation maintained in any one population. Populations of ruderal species like *M. annua* are often very recently colonised and intrinsically ephemeral in nature. While we should expect selection to operate at the population (or deme) level for many traits, life-history and reproductive strategies of ruderal species will often be under the influence of selection brought about by processes of colonisation and extinction, as well as fluctuations of density, that are germane to metapopulations. The responses to selection that we have observed in our experiment are thus best interpreted in terms of what might be expected over the longer term as a result of such processes over large spatial scales, in particular the repeated stochastic loss of males from populations as a result of colonisation bottlenecks.

An important implication of our sampling design was also to allow natural selection each of our replicate populations to act on what was likely to be a large level of standing genetic variation, which was probably at least partly responsible for the high level of variation in the leakiness expressed by females in generation 0. The number of male flowers produced by females from generation 0 in the common garden ranged from zero to 198 flowers on a single individual, with a mean MRE of 6.36×10^{-5} ($SD = 5.44 \times 10^{-4}$). The MRE of females corresponds to what we termed in Chapter 3 the inconstant reproductive effort (λ) of females. The values recorded from the base population in the common garden are comparable to those measured in Chapter 3 which were drawn from the same genetic base.

Phenotypic plasticity in leaky sex expression

Even in the first generation of our experiment, before any response to selection, variation of MRE differed significantly between control and selected lines in the field, with females in male-less plots producing more male flowers than those in plots with 50% males. This hints at a plastic component of MRE in females of *M. annua*. We did not record abiotic environmental conditions, so we cannot rule out the possibility that the difference in male flower production we observed in leakiness in the first generation was a response to the different environments experienced among plots. However, given that the mating environment differed strongly between treatments, the enhanced leakiness of male-deprived females was very likely a plastic response to the severe pollen limitation that they experienced. Sex allocation is often a plastic trait in plants in general (Korpelainen, 1998), and plastic responses to mate limitation have previously been recorded in a number of species (Albert *et al.*, 2011; Jones *et al.*, 2013). While plastic sex allocation is likely to buffer the response to selection on sex-allocation traits (West-Eberhard, 2003; Crispo, 2008), environmentally induced phenotypes may reveal cryptic genetic variation to natural selection, so that plasticity might actually enhance the potential for trait evolution (Pigliucci *et al.*, 2006; Crispo, 2007; Ghalambor *et al.*, 2007; Schlichting, 2008). The potential role of phenotypic plasticity in facilitating selection among diverging phenotypes awaits further analysis.

Causes of the rapid phenotypic shift in male flower production by females

In what way did the removal of males from females favour increased inconstancy in our experiment? There would seem to be two modes of selection that might have acted. First, females might have benefited from leaky sex expression through reproductive assurance, via their capacity to self-fertilize. In a metapopulation with frequent colonisation of new habitat by potentially single females, an ability to self-fertilize would constitute a substantial advantage in assuring their seed production. Indeed, this process has long been invoked to explain the maintenance of a male function in monoecious individuals of tetraploid and hexaploid *M. annua* (Pannell, 2000). In the first generation of our experiment, where seed production in male-less replicates was substantially pollen-limited, we expect sex-inconstant females self-fertilized a sizable fraction of their seeds, and thus likely benefited from their pollen production through assurance of their female function. However, the relative importance of this mode of selection likely declined in subsequent generations of our experiment, replaced by a second, greater advantage of male function.

The second, likely more important, benefit of the evolved male function of inconstant females of selection arises from their ability not only to self-fertilize some of their own ovules, but to compete with other females in the population to sire the many ovules that the population of females as a whole has produced. We do not yet know what the selfing rates were in the experimental

populations, but we expect that they were low to negligible, at least in the later generations when many females were already producing pollen. Thus, a given female with a leaky male function would expect to sire, on average, a fraction of the ovules in the population proportional to the fraction of pollen she contributed to the outcross pollen pool. Under this type of 'mass action' mating (i.e., mating under lottery-model competition, which is appropriate for a wind-pollinated herb; Charlesworth & Charlesworth, 1981; Charnov, 1982; Hesse & Pannell, 2011), a shift in allocation female to male flowers would confer a potentially enormous selective advantage. We thus believe that this second form of selection may mainly drive the evolutionary changes we have observed, because we grew each experimental population in high densities favouring outcrossing over selfing, as previously recorded in *M. annua* (Eppley & Pannell, 2007).

Selection under mate limitation in dioecious species in which both sexes are inconstant may in principle act on either males or females, to bring about shifts away from dioecy. In the study of *Leptinella* species by Lloyd (1975a), monoecious populations derived from dioecious ancestors derived either from females (the heterogametic sex in *Leptinella*) or males. The author showed that male-derived populations were more likely to establish stable monoecious populations because the first bout or reproduction of the homogametic sex will not immediately re-establish a 1:1 sex ratio in the progeny. We show in the present study that similar shifts in sex allocation are possible in *M. annua* when females are isolated from males, even when they constitute large populations. It would be interesting to test if males placed under the same conditions as our selected females would increase their female allocation as fast as what we observed in females. Such male-only populations may alternatively be driven towards what Lloyd (1975a) termed 'pseudo-monomorphic dioecy', to describe the populations most likely derived from isolated *Leptinella* females, characterized by biased sex-ratios and higher inconstancy than in dioecious populations.

Heritability of inconstancy in females of *M. annua*

Our results suggest that there is a heritable genetic component of female inconstancy in *M. annua*. We estimated the heritability of inconstant male allocation of females over the four generations of of our experiment to be approximately 0.73 (*SD* = 0.48), averaged across generations. The measured heritability of MRE in females compares with traits for which the highest values of heritability have previously been inferred in a number of flower related traits in natural populations of other angiosperm species (e.g., Mitchell & Shaw, 1993; Campbell, 1996; Johnson et al., 2009). However, our calculation of heritability may contain flaws that must be considered. In particular, at the fourth generation, our estimate of heritability is greater than 1, which is theoretically impossible. First, our measure of heritability was not based on parent-offspring regression, as is usually the case

(e.g., Keller et al, 2001), but derived directly from the response to selection observed and estimates of the selection differential, both of which are measured with error.

Our calculations of selection differentials were based on the covariance between MRE and relative fitness. The calculation of relative fitness can introduce difficulties in estimates of selection differentials: it only takes into account the relative male and female fertility of each individual compared to the whole population, and does not include measures of progeny survival. Indeed, germination rates were variable across replicates and generations, and may have affected our results if one particular mother produced numerous seeds but only a minority that germinated when we set up the following generation. Second, the observed phenotypic response to selection is the sum of genetic and environmental effects on these phenotypes. If individuals of the progeny are environmentally correlated, this could drive the response to selection and lead to estimates of heritability greater than 1. There was substantial variation between years in the biomass of females, probably hinting at different environments experienced by population between generations, potentially impacting our measures of response used to estimate heritability. Although our heritability estimates may have been biased by these processes, it is clear that heritability of leakiness in our experiment was high.

Implications for sexual-system transitions in plants

To what extent might the dramatic increase in pollen production by females of *M. annua* be interpreted as representing the beginning of a shift in sexual system? The answer to this question depends very much on the context in which the new phenotypes find themselves. Even though there was a substantial expression of leaky male function in females in the base population, seed production by plants in the male-deprived populations was strongly pollen-limited. However, after the first generation, signatures of pollen limitation disappeared with the increased pollen production of the females, and the high seed set by their individuals indicate that such male-less populations are likely to be viable under wild conditions. In this sense, it seems fair to conclude that the response to selection we observed do indeed represent a qualitative shift away from dioecy. Given that a substantial number of individuals in these populations continued to lack a male function and were completely female in function, it is tempting to consider the evolved populations gynodioecious. However, the distribution of male flower production in these populations was continuous and not bimodal, so that gynodioecy would likely be a misleading term. Rather, it might be more appropriate to consider them ‘sub-monoecious’.

Given that *M. annua* typically occurs in a metapopulation in the wild, in which monoecious (or 'sub-monoecious' population might occur in a landscape matrix with populations containing males (Pannell, 1997Evol), what might occur in an evolved population upon the immigration of males? This process is thought to be common in androdioecious regions of the hexaploid populations of *M. annua*, where monoecious populations can be established by self-fertile monoecious individuals into which males can later invade and spread to intermediate frequencies (Pannell, 2000). The possession of a substantial male function by monoecious individuals in these hexaploid populations means that frequencies invading males can reach are lower then 50%; indeed, it is this lower frequency of males that qualifies these populations as functionally androdioecious rather than dioecious. Could the male function that has evolved in the leaky females of our experiment be sufficient to prevent the frequency of re-invading males from reaching 50%?

The equilibrium frequency of males in a population of pollen-producing females (or hermaphrodites) is given by the expression

$$p=\frac{r\alpha(1-s)-2(1-s\delta)}{2(r\alpha-1)(1-s\delta)}$$

(modified from Pannell, 2000), where r is the amount pollen produced by males relative to hermaphrodites, α is the relative outcrossing performance of pollen produced by males relative to that of hermaphrodites, s is the rate of self-fertilisation of the hermaphrodites, and δ is the level of inbreeding depression suffered by self-fertilized progeny. Eppley & Pannell (2009) estimated $\delta = 0$ for western populations of diploid *M. annua*, a value that is perhaps due to the purging effects on inbreeding depression by the range expansion of *M. annua* from eastern Europe (Pujol *et al.*, 2009). Eppley & Pannell (2007) estimated s to be low for populations of *M. annua* at high density; given the high densities of our experiment, we might conservatively suppose that $s = 0$. Eppley and Pannell (2007) also estimated λ to be 1.6, i.e., pollen grains produced by males have a 60% greater chance of successfully fertilizing an outcrossed ovule than those produced by hermaphrodites. Finally, we may estimate r as equal to the reciprocal of the d, as set out in Chapter 3, i.e., the reciprocal of the allocation of leaky females or hermaphrodites relative to that of males. Taking the value of $d = 0.019$ from our experimental females after four generations of selection, and substituting these values in the expression above, we find that males should reach an equilibrium frequency of 0.49. This is very close to 0.5. For populations at high density, therefore, we conclude that populations comprising males with females with the evolved increased pollen production from our experiment would still be best regarded as functionally dioecious.

Because *M. annua* populations are known to fluctuate in density, and that density directly determines the selfing rate of hermaphrodites (Eppley & Pannell, 2007) and presumably females with leaky sex expression, we might ask what the sexual system of a population of males with evolved females at low density might be, i.e., where the selfing rate is substantial. For example, assuming that s = 0.5, which seems reasonable for *M. annua* (Korbecka *et al.*, 2011), the equilibrium frequency of males would be 0.24, i.e., substantially lower than 0.5. Such populations would thus have an androdioecious sexual system, in which the evolved females contribute substantially through both their male and female functions.

The density-dependence of selfing rates in *M. annua* (Hesse & Pannell, 2011a) should strongly influence the establishment of androdioecy, as demonstrated by Dorken & Pannell (2008). The persistence of sexual systems seems thus directly related to density and population size in *M. annua*. It is possible that androdioecy was never established in the dioecious lineage because of their large population size, allowing males to be maintained at high frequency, and selecting for a strongly female-biased allocation in hermaphroditic individuals, i.e., for functional females. This may indicate that inconstancy in females is in fact a relict of a past monoecious state of these individuals, which may have once been favored during periods of low population density, or during range expansions (Obbard *et al.*, 2006b). Our selection experiment showing a rapid increase in sex allocation in response to low male frequency certainly points in that direction. If changes in the sexual system of *M. annua* are density-dependent, as we suppose, and as has been shown for polyploid populations, we would expect that geographic isolation might be associated with transitions in sexual system, from separate sexes to combined sexes. We would then expect transitions towards dioecy to be rarer than transitions away from dioecy, a pattern recently highlighted by several phylogenetic studies (Käfer *et al.*, 2014, 2017; Sabath *et al.*, 2016).

Reversions from dioecy to hermaphroditism have long been suggested to have occurred in the evolutionary history of angiosperms (Lloyd, 1975a; Wolf & Takebayashi, 2004; Renner, 2014; Käfer *et al.*, 2017), which may explain the scattered distribution of dioecy among flowering plant species (Renner, 2014). Among other potential causes (reviewed in Kafer *et al.*, 2017), pollen limitation is likely to be a major factor favouring hermaphroditism and the breakdown of dioecy (Wolf & Takebayashi, 2004; Crossman & Charlesworth, 2014). Our present study illustrates the potential for sex inconstancy to bring about such transitions, and might represent the first evolutionary step of a breakdown of dioecy through selection under conditions of mate limitation. The two recent theoretical studies that focused on sexual-system evolution away from separate sexes directed their attention on inconstant males (Ehlers & Bataillon, 2007; Crossman &

Charlesworth, 2014), mainly because males are more frequently found inconstant than females in natural dioecious populations (Ehlers & Bataillon, 2007; Chapter 3). Models show that reversions are possible under particular conditions - including pollen limitation, a recent evolution of dioecy, and low levels of inbreeding depression - and that it may result in either a complete reversion to hermaphroditism, or to the coexistence of modified males with a female function and canalized females, i.e., gynodioecy (Ehlers & Bataillon, 2007; Crossman & Charlesworth, 2014). Our study here shows that shifts from dioecy to hermaphroditism may also occur by selection on female inconstancy. Prevalent female inconstancy might be particularly relevant for species that have evolved separate sexes via a monoecious intermediate step like *M. annua* (Chapter 3). This would be in contrast with other dioecious species, like *Silene latifolia* or *Fragaria virginiana*, that are likely to have followed a gynodioecious path to dioecy via the spread of male sterility mutations; it would seem that monoecy and/or androdioecy are less likely to arise as a result of the breakdown of dioecy in such species.

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FIGURES

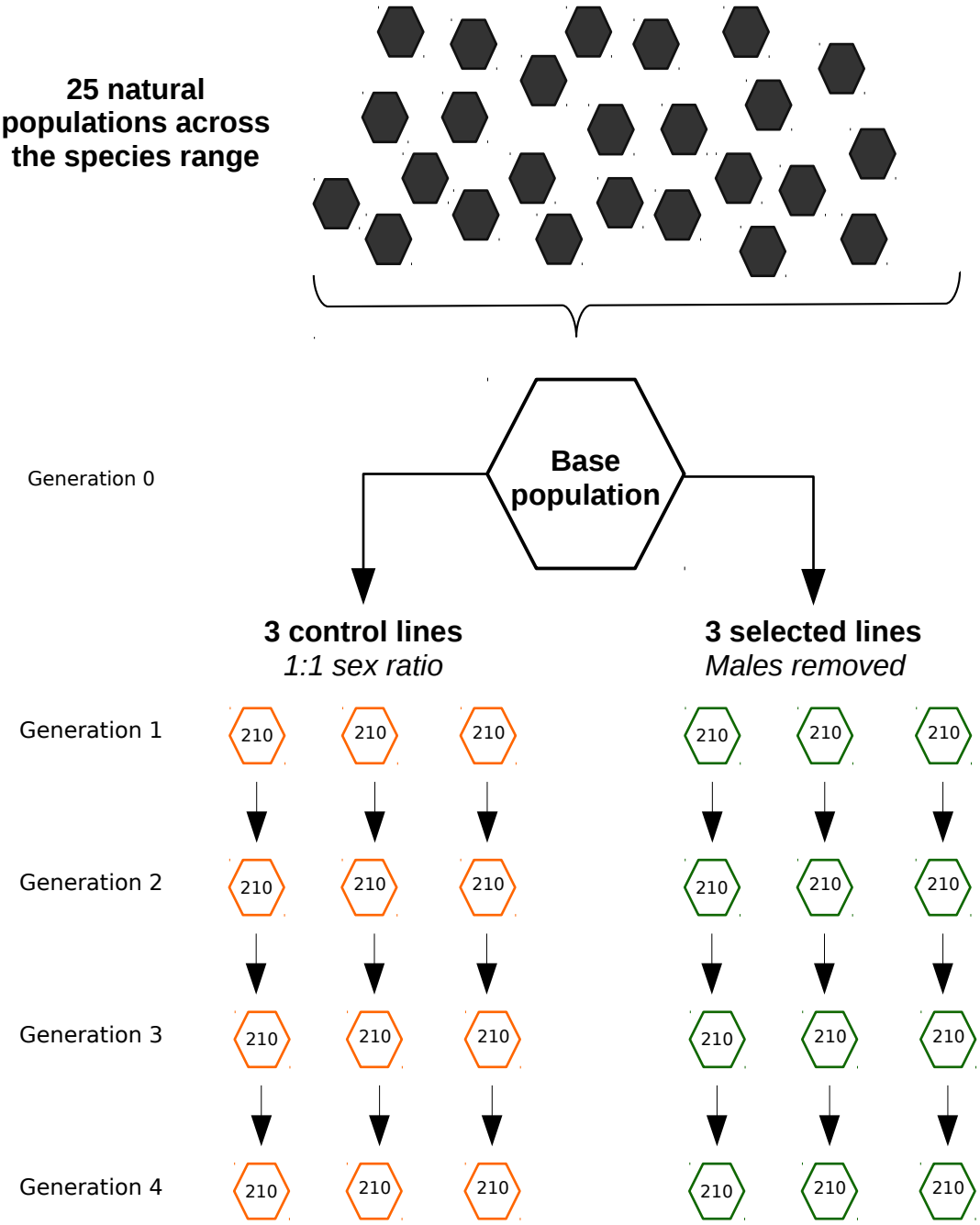

Figure 1. Experimental design of the selection experiment. Black hexagons represent natural populations of *M. annua*. Orange hexagons represent experimental populations of the control lines. Green hexagons represent experimental populations of the selected lines. Each experimental line was constituted of 210 individuals. The base population was constituted of a seed pool taken from 25 different natural populations. Experimental lines were allowed to evolve independently. Seeds from each line were used to constitute the following generation of the same line (represented by arrows). Seeds from each line at each generation were sown in a common garden after the 4th generation. Field data were measured on adult plant growing at a given generation. Common garden data were measured on seedlings issued from a given generation.

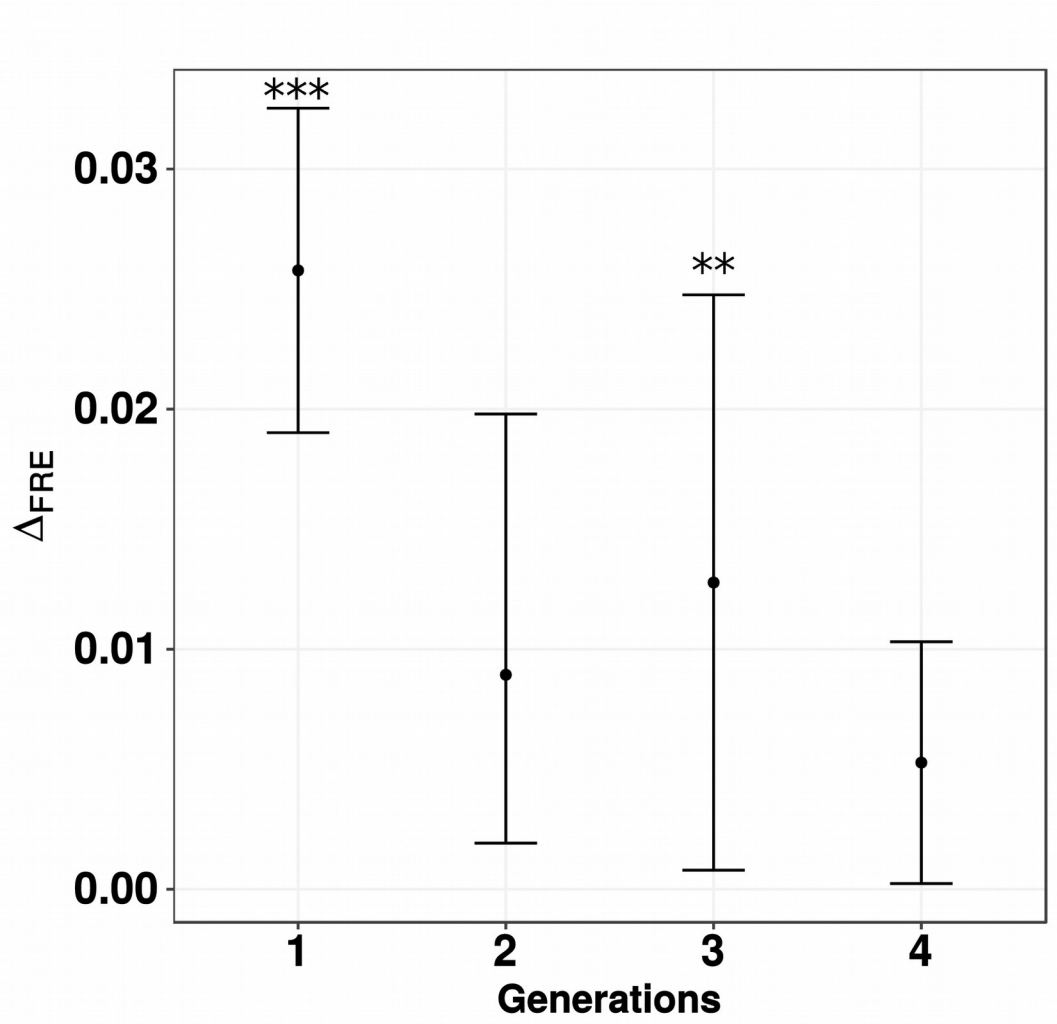

Figure 2. Mean difference in female reproductive effort (Δ_{FRE}) between females of control and selected lines. Whiskers indicate 95% confidence intervals. Stars indicate a significant difference of the mean, tested using Mann-Whitney rank tests, ** : $0.001 < P < 0.01$; *** : $P < 0.001$.

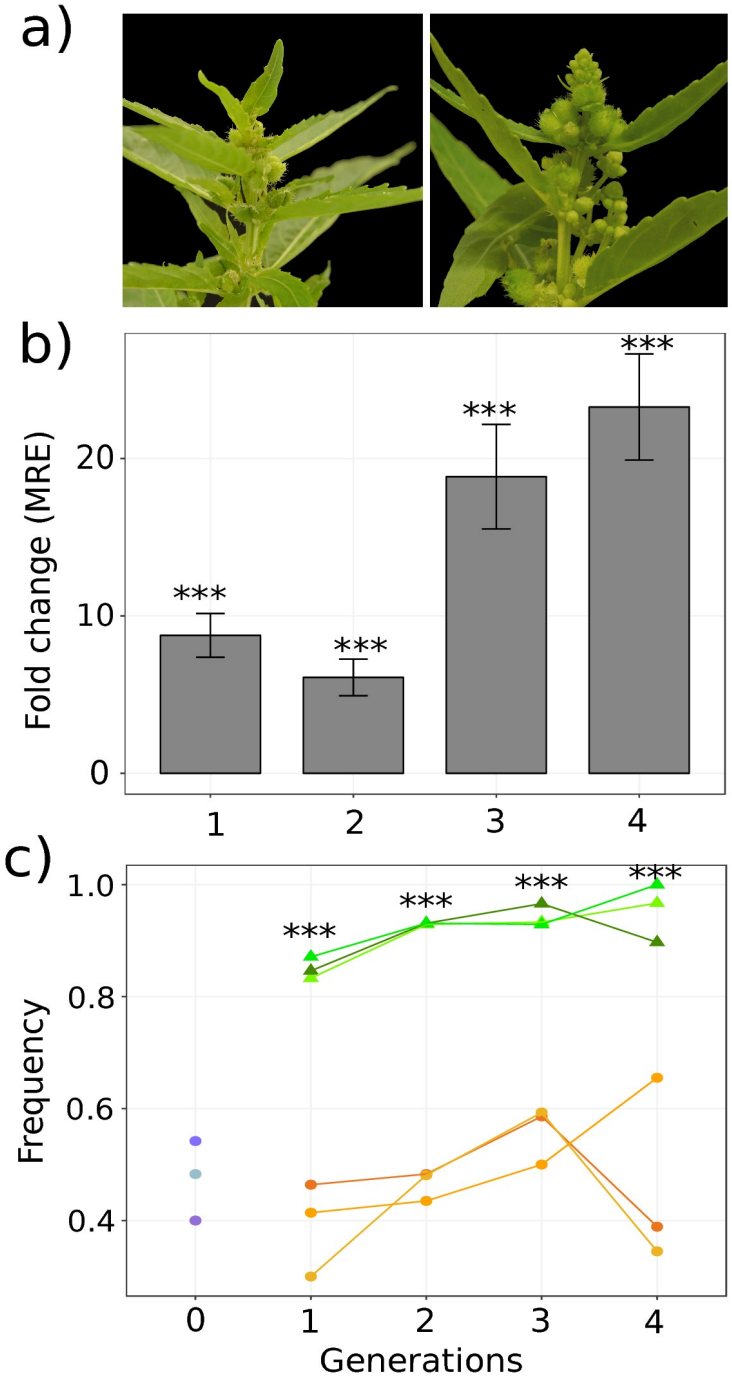

Figure 3. Response to selection in selected lines compared to control lines, measured from common garden. **(a)** Photographs of typical female from the control lines (left) and modified female from selected lines (right). **(b)** Fold change of male reproductive effort (MRE) between females of control and selected lines, averaged per replicates, per generation. Whiskers represent standard errors. Stars indicate significant differences in MRE between control and selected lines (Mann-Whiney ranked tests). **(c)** Frequency of females above the mean MRE of females in initial populations (purple), of 1.58 $x10^{-4}$) in selected (green) and control lines (orange). Stars indicate significant difference between control and selected lines, tested using a generalized linear model with a binomial distribution, , * : $0.01 < P < 0.05$; *** : $P < 0.001$.

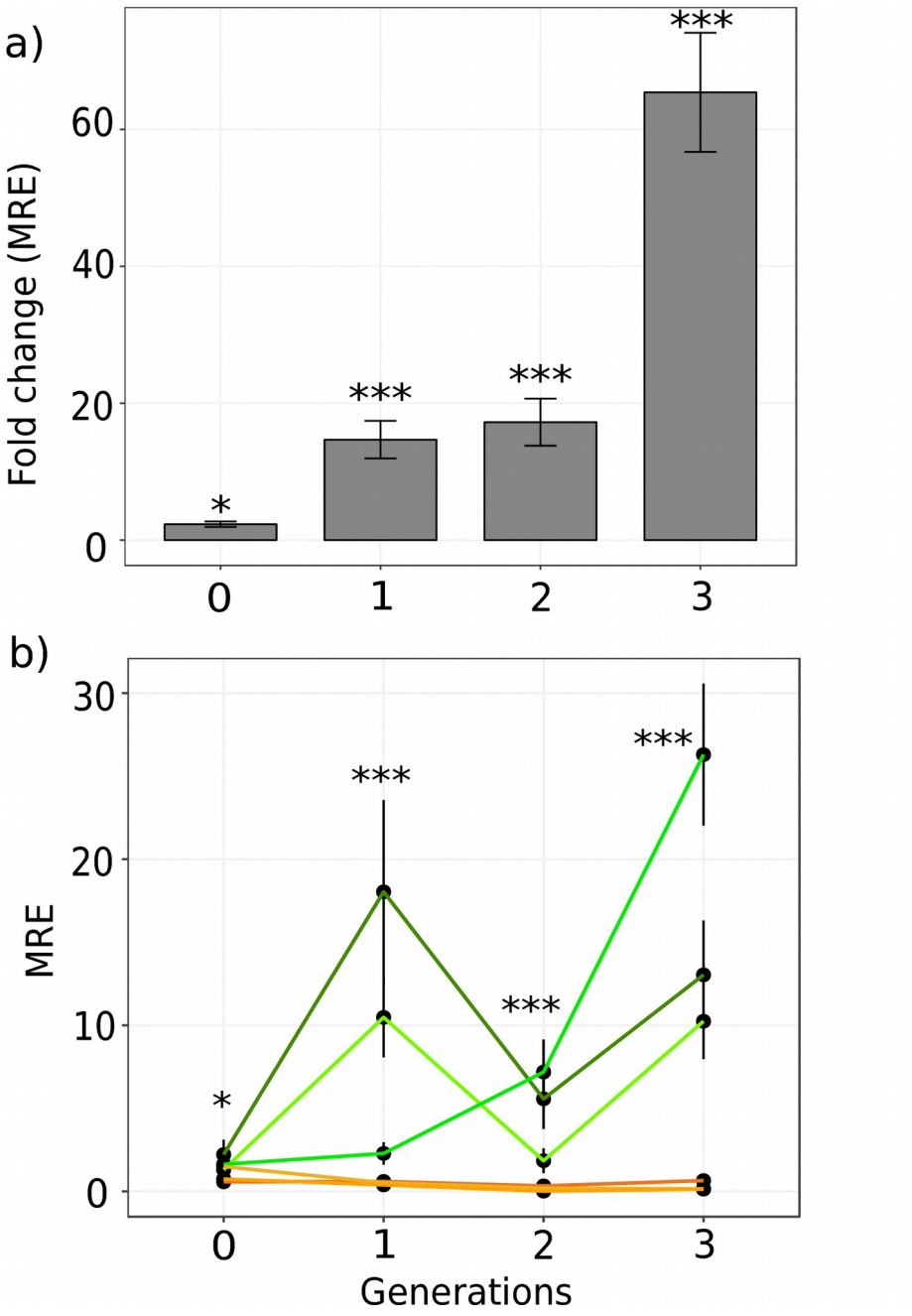

Figure 4. Phenotypic evolution under pollen limitation, measured in the field. **(a)** Fold change of male reproductive effort (MRE) between females of control and selected lines, averaged per replicates, at each generation. Whiskers represent standard errors. Stars indicate significant differences in MRE between control and selected lines (Mann-Whiney ranked tests). **(b)** Mean MRE of females in each replicate of the selected (green) and control (orange) lines. Whiskers indicate standard error. Stars indicate significant difference between control and selected lines (Mann-Whiney ranked tests), * : $0.01 < P < 0.05$; *** : $P < 0.001$.

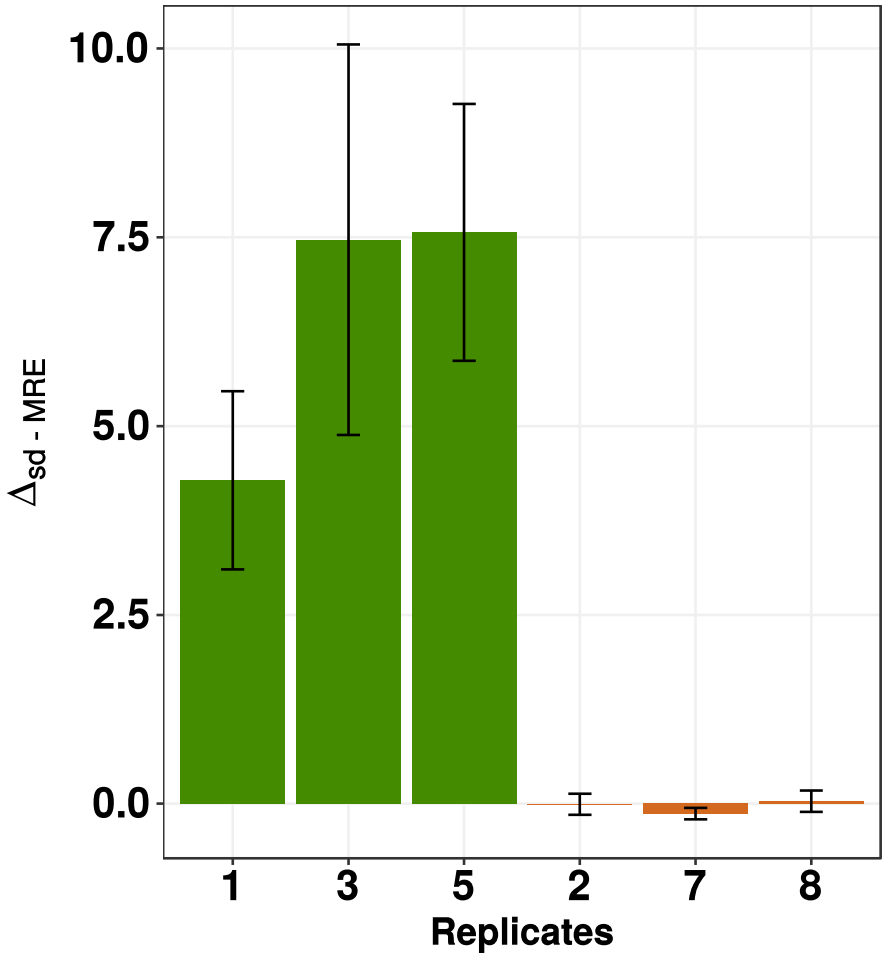

Figure 5. Response to selection of male reproductive effort (MRE), expressed in the number of standard deviation for MRE present in the initial generation ($\Delta_{sd\text{-}MRE}$), in selected (green) and control (orange) lines. Whiskers represent standard error.

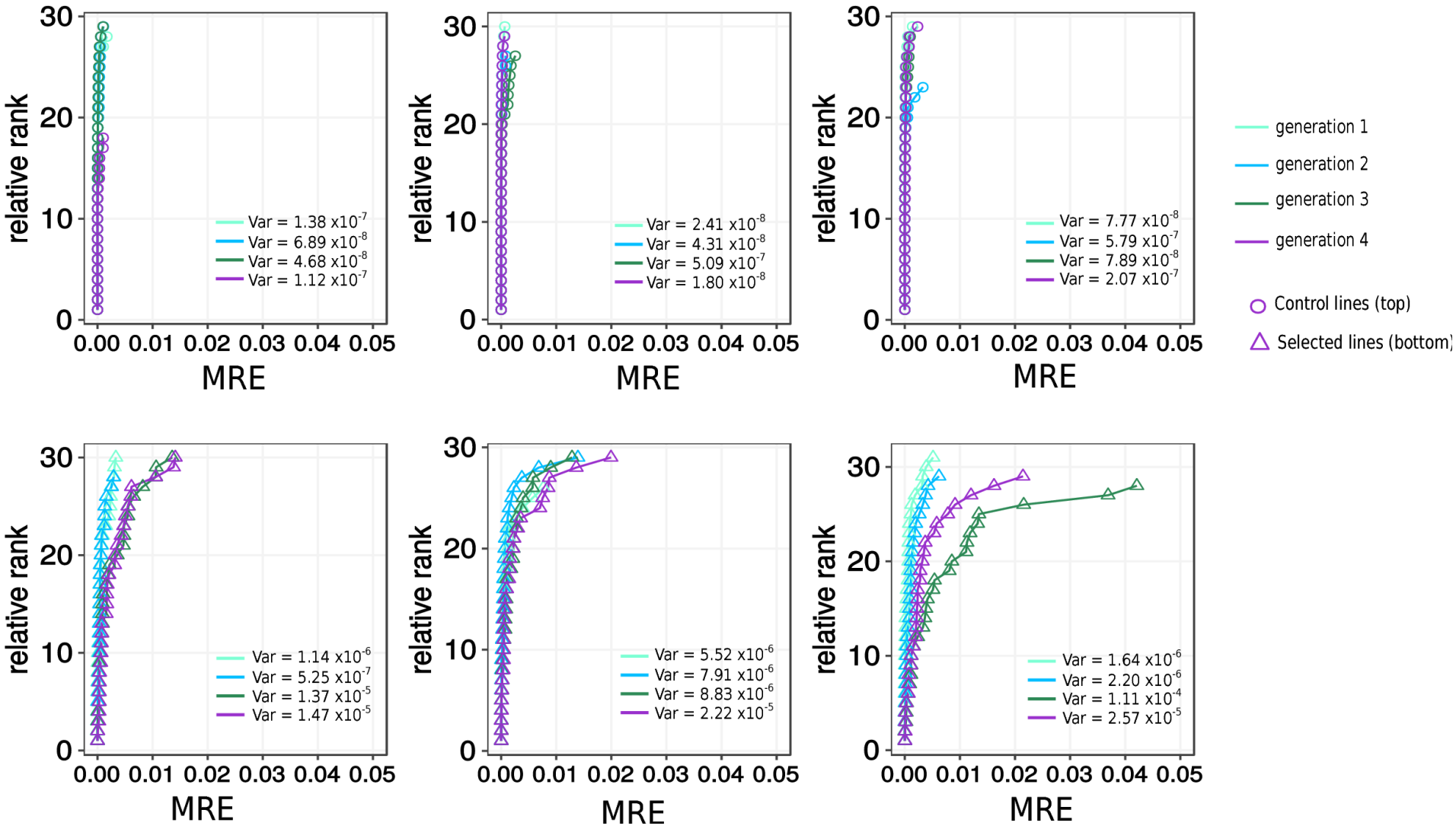

Figure 6. Distribution of male reproductive effort (MRE), in control (top graphs) and selected lines (bottom graphs), measured in the common garden, plotted per order of its rank among the sampled plants of each experimental populations. Sample variance per generation is indicated for each experimental population.

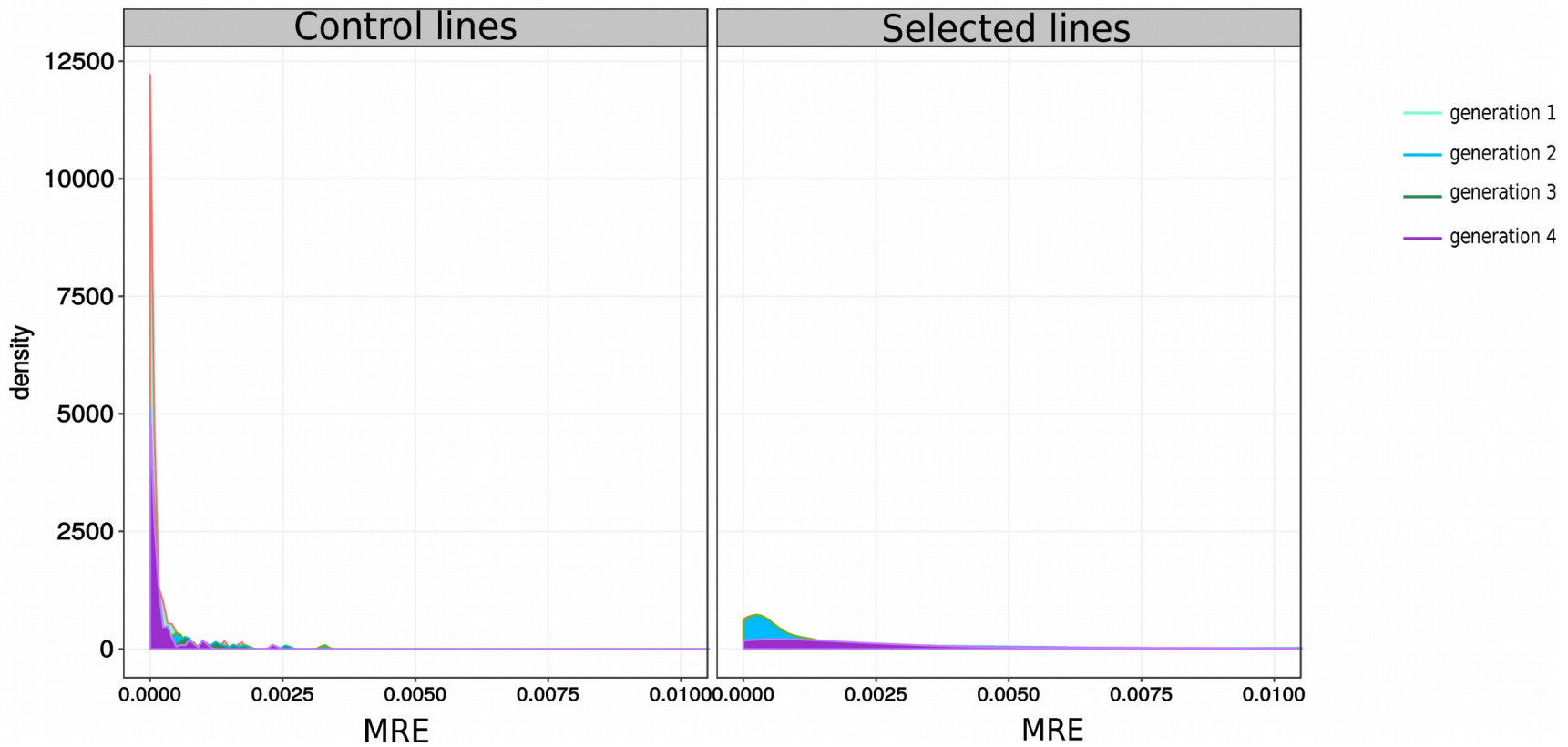

Figure 7. Density distribution of male reproductive effort (MRE), in control and selected lines, measured in the field. Each colour correspond to a generation of selection. Control lines conserved distribution close to zero, selected lines showed a shift of the distribution of MRE towards an increased mean value, with an almost gaussian shape for the two first generations, which becomes almost uniform at generations 3 and 4.

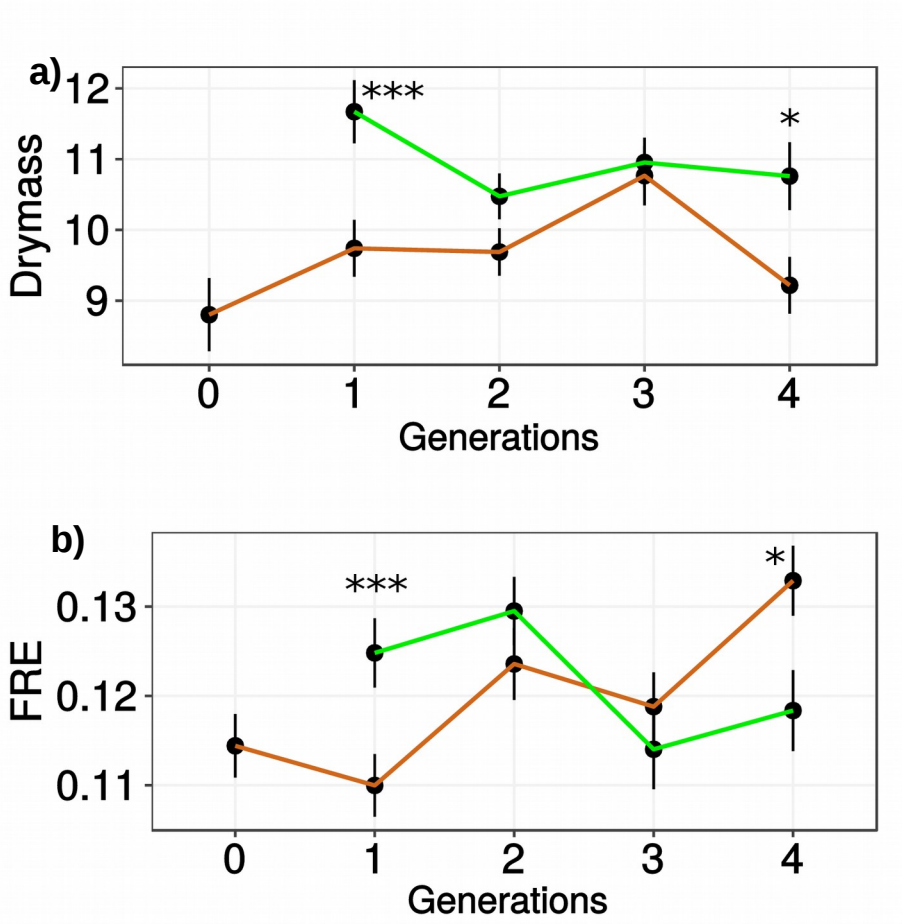

Figure 8. Tracking of **(a)** above-ground drymass (in *g*) and **(b)** female reproductive effort (FRE), averaged per replicate, in selected (green) and control (orange) lines, measured in a common garden. Whiskers indicate standard error. Stars indicate significant difference between control and selected lines (Student's *t*-tests), * : $0.01 < P < 0.05$; *** : $P < 0.001$.

TABLES

Table 1. Means and standard deviations of phenotypic traits, in field populations, measured on females. **N**: sample size. **V**: height /drymass. **MRE**: male reproductive effort (mass of male flowers/drymass). **FRE**: female reproductive effort (mass of seeds/drymass). Wilcoxon tests (two-sided) have been used for statistical comparisons between control and selected females.

Generation	**1**			**2**			**3**			**4**		
Treatment	50% males	Males removed	*P*	50% males	Males removed	*P*	50% males	Males removed	*P*	50% males	Males removed	*P*
N	113	159	-	149	148		148	150	-	150	150	-
Drymass (g)	4.68 +/- 2.32	3.59 +/- 1.35	0.56	7.20 +/- 3.20	8.62 +/- 4.56	0.48	5.42 +/- 2.04	8.84 +/- 9.42	0.13	8.06 +/- 3.73	7.21 +/- 3.40	0.69
V (cm/g)	NA	NA	NA	13.74 +/- 7.30	13.32 +/- 9.07	0.92	13.09 +/- 4.83	11.82 +/- 5.50	0.33	12.77 +/- 7.01	11.94 +/- 5.96	0.66
MRE ($x10^{-4}$)	1.94 +/- 4.14	4.48 +/- 9.69	4.7×10^{-3} **	8.27 +/- 3.12	12.1 +/- 21.0	$< 1.4 \times 10^{-3}$ **	0.39 +/- 1.55	6.72 +/- 14.6	5.00×10^{-4} ***	0.40 +/- 1.04	25.9 +/- 35.7	2.1×10^{-3} **
FRE ($x10^{-2}$)	7.41 +/- 2.19	4.97 +/- 2.67	0.25	8.27 +/- 3.20	7.37 +/- 3.26	0.81	13.1 +/- 4.83	11.8 +/- 5.50	0.33	5.50 +/- 2.01	4.97 +/- 2.22	0.50

* : $0.01 < P < 0.05$; ** : $0.001 < P < 0.01$; *** : $P < 0.001$

Table 2. Means and standard deviations of phenotypic traits in common garden, measured on females. **N**: sample size. **V**: height /drymass. **MRE**: male reproductive effort (mass of male flowers/drymass). **FRE**: female reproductive effort (mass of seeds/drymass). ***d:*** degree of inconstancy (with standard errors). Wilcoxon tests (two-sided) have been used for statistical comparisons between control and selected females.

Generation	**0**	**1**			**2**			**3**			**4**		
Treatment	50% males	50% males	Males removed	*P*	50% males	Males removed	*P*	50% males	Males removed	*P*	50% males	Males removed	*P*
N	73	87	87	-	79	86	-	84	87	-	76	88	-
Drymass (g)	8.80 +/- 4.41	9.80 +/- 2.95	11.69 +/- 3.49	2.34 $x10^{-3}$ ***	9.68 +/- 2.97	10.47 +/- 2.95	0.055	10.74 +/- 3.67	10.95 +/- 3.30	0.60	8.99 +/- 3.14	10.79 +/- 3.86	0.03 *
V (cm/g)	8.43 +/- 4.14	7.34 +/- 2.16	5.98 +/- 1.90	6.86 $x10^{-4}$ ***	6.93 +/- 1.91	6.39 +/- 1.88	0.049 *	6.48 +/- 2.07	6.19 +/- 1.70	0.84	7.33 +/- 2.15	6.86 +/- 2.71	0.11
MRE ($x10^{-4}$)	1.58 +/- 5.44	1.10 +/- 2.69	9.66 +/- 14.1	2.55 $x10^{-13}$ ***	1.81 +/- 4.10	10.6 +/- 16.7	2.03 $x10^{-13}$ ***	2.27 +/- 4.04	42.6 +/- 57.3	< 2.2 $x10^{-16}$ ***	1.48 +/- 3.08	33.3 +/- 45.4	< 2.2 $x10^{-16}$ ***
FRE	0.11 +/- 0.031	0.11 +/- 0.030	0.13 +/- 0.036	8.15 $x10^{-3}$ ***	0.12 +/- 0.036	0.13 +/- 0.035	0.31	0.12 +/- 0.034	0.11 +/- 0.041	0.61	0.13 +/- 0.033	0.12 +/- 0.042	0.022 *
d ($x10^{-3}$)	0.90 +/- 3.08	0.68 +/- 1.73	5.92 +/- 8.76	2.55 $x10^{-13}$ ***	1.02 +/- 26.5	6.21 +/- 11.0	2.03 $x10^{-13}$ ***	13.4 +/- 27.9	25.2 +/- 41.4	< 2.2 $x10^{-16}$ ***	0.83 +/- 1.94	19.2 +/- 261	< 2.2 $x10^{-16}$ ***

* : $0.01 < P < 0.05$; ** : $0.001 < P < 0.01$; *** : $P < 0.001$

Table 3. Standardized univariate linear (β) and quadratic (γ) selection gradients showing directional on the male reproductive effort (MRE) of females grown in the absence of males.

Generation	Treatment	β	γ
1	50% males	0.087 (0.048)	- 0.0095 (0.084)
	no males	**0.81 (0.043) *****	**0.284 (0.052) ****
2	50% males	0.056 (0.102)	- 0.003 (0.046)
	no males	**0.73 (0.070) *****	0.131 (0.191)
3	50% males	9.53 $x10^{-3}$ (9.33 $x10^{-3}$)	- 6.41 $x10^{-3}$ (0.016)
	no males	**0.89 (0.038) *****	**0.22 (0.039) ****
4	50% males	1.33 $x10^{-3}$ (0.049)	-0.057 (0.095)
	no males	**0.72 (0.109) *****	0.151 (0.062)

Estimates are average linear (β) and quadratic (γ) selection gradients calculated separately for each experimental population. Standard errors are in parentheses. Stars indicate values that are significantly different from zero at p = 0.05, calculated from a comparison of the mean selection-gradient values and the null expectation of zero via t-tests with 2 degrees of freedom (i.e., the experimental populations served as the unit of observation). ** : 0.01 < P < 0.05 ; ** :* 0.001 < *P* < 0.01 *; *** : P* < 0.001

- CHAPTER 5 -

Changes in sex-biased gene expression associated with selection for increased male allocation in females *Mercurialis annua*

Guillaume Cossard and John R. Pannell

Introduction

Plant and animal species with separate sexes are characterized by phenotypic divergence between genders. This includes of course structures primarily involved in reproduction, but can also involve secondary traits not directly related to reproduction (Lloyd & Webb, 1977), resulting in secondary sexual dimorphism (SD). While SD is widespread in animal species (Darwin, 1874; Shine, 1989), in which it sometimes reaches extreme levels (e.g., Vollrath 1998), it is less conspicuous although frequent in plants (Lloyd & Webb, 1977; Barrett & Hough, 2012).

Males and females may have different genetic interests, and selection often acts differently on them, driving their phenotypic divergence. This is often theoretically represented by differing optimal strategies between the sexes, towards which both sexes are driven by adaptive shifts (Connallon & Clark, 2010). Disruptive selection that drives each sex to its own fitness optimum acts mainly upon a shared genome between sexes, with the potential exception of the sex determining region (SDR), located on sex chromosomes (Charlesworth, 2002, 2013; Wright *et al.*, 2016). Autosomal, and pseudo-autosomal (PAR) regions in species with sex chromosomes, thus may harbour genes that are beneficial to one sex but detrimental to the other. These sexually antagonistic (SA) genes are thought to be the source of intra-locus sexual conflicts (Chapman *et al.*, 2003; Prasad & Bedhomme, 2006). One classical example of sexual antagonism is the case of male ornamentation in guppies, in which coloration patterns of males favour their attractiveness and mating success while it simultaneously increases the predation risks in both sexes. Studies have demonstrated that most of the variation of ornamentation in guppies is encoded by Y-linked genes (Brooks, 2000; Brooks *et al.*, 2001). Shared loci under sexually antagonistic pressures may show genetic correlations between sexes, thus constraining the extent to which sexual dimorphism can evolve (Griffin *et al.*, 2013).

The evolution of sexual dimorphism is only possible through some degree of resolution of intra- and inter-locus sexual conflicts (Bonduriansky & Chenoweth, 2009), either by sex-linkage of SA genes, which tend to accumulate in the vicinity of the sex-determining loci (Charlesworth & Charlesworth, 1978a; Rice, 1984), by gene duplication and subsequent sex-specific differentiation of paralogs (Connallon & Clark, 2011), by genomic imprinting (Day & Bonduriansky, 2004), or by the evolution of differential expression in the two sexes depending on sex-specific optima (Ellegren & Parsch, 2007; Parsch & Ellegren, 2013), and which lead to sex-biased gene expression (SBGE). SBGE is widespread in animal species, in which the level of differential expression between sexes strongly depends on the tissue investigated (Meisel et al. 2012; see Table 6, Chapter 2 for review), and on genetic correlations between sexes.

Sexual conflicts can also arise within the genome of hermaphrodites (Jordan & Connallon, 2014). Such conflicts are expected to show different patterns than those found for separate sexes (Bedhomme *et al.*, 2009), because neither sex linkage nor sex-specific regulation of sexual functions for intra-locus sexual conflicts can evolve in hermaphrodites. The absence of sex-specific regulation of SA genes exposes these loci to selection during each generation, while genes that show sex-specific expression will on average be expressed in half of individuals (given a balanced sex-ratio), reducing the effects of selection. This may result in quicker allele-frequency changes of SA genes, leading to a higher probability of fixation or loss in hermaphroditic species compared to dioecious ones (Reinhold, 2000; Bedhomme *et al.*, 2009). In addition, mating-system changes may occur in hermaphroditic populations, sometimes involving higher levels of selfing, which is known to differentially affect the outcome of SA selection (Jordan & Connallon, 2014).

The evolution of SBGE is still poorly understood. Indeed, it is difficult to assess whether the patterns of SBGE we observe in natural populations reflect fully resolved sexual conflicts, so that each sex has reached an optimal expression level for a particular gene, leading to the suppression of sexual conflicts. An experimental study has recently uncovered on-going unresolved sexual conflicts that constrain the evolution of SBGE and SD in natural populations of *Drosophila melanogaster* (Hollis *et al.*, 2014). In that study, the experimental manipulation of mate availability for females, by imposing monogamy in selected lines, supposedly relaxed sexual selection acting *via* male-male competition and female-choice. After 65 generations of evolution under monogamy, both male and female transcriptomes showed significant signs of feminization, i.e., an increased expression of female-biased genes, suggesting that gene expression in both sexes is probably kept at a sub-optimal level in natural populations as a result of a compromise struck between the sexes (Hollis *et al.*, 2014).

The evolutionary causes of SBGE still largely need to be elucidated (Parsch & Ellegren, 2013). Indeed several processes may lead to the evolution of SBGE: sexual antagonism, when selection acts in opposite direction in sexes, is often invoked as a cause of SBGE (Grath & Parsch, 2016). Alternatively, selection for increased expression in females and stabilizing selection in males, as well as selection for decreased expression in males and stabilizing selection in females, would both lead to female- and male-biased gene expression, respectively. Finally, primary changes of expression, associated with sex-specific developmental pathways and pleiotropic effects associated with sex-biased expression at other loci, may also lead to sex-biased gene expression, although not directly underlined by sexual conflicts.

The recent evolution of separate sexes in plants may help to shed light on the evolutionary causes of SBGE. Separate sexes in plants has most likely evolved from an hermaphroditic ancestor, either via the spread of sterility mutations (Charlesworth & Charlesworth, 1978a), leading to a fixed expression of gender, or through gradual shifts of sex allocation in each of the male and female gender classes, leading to a bimodal distribution of gender, that tend to be leaky (see Chapter 3; D. Charlesworth and Charlesworth 1978; Charnov 1982). SBGE has previously been observed in dioecious plants, in reproductive tissues (Liu *et al.*, 2013), as well as in vegetative tissues (Zluvova et al., 2010; Robinson et al., 2014; Harkess et al., 2015; Zemp et al., 2016; Chapter 2). A recent study has investigated the dynamic of SBGE evolution during the transition from gynodioecy towards separate sexes (Zemp *et al.*, 2016). In that study, patterns of expression of sex-biased genes in both leaves and flowers were compared between dioecious *Silene latifolia* and gynodioecious *Silene vulgaris*. The authors concluded that SBGE in the dioecious species had arisen mainly from selection for down-regulation of genes in females (Zemp *et al.*, 2016), supporting the idea that sexual antagonism was present within the genome of the hermaphroditic ancestor and that the resolution of these intralocus sexual conflicts may have driven the evolution of SBGE in dioecious *S. latifolia.*

It is likely that the evolution of SBGE is also constrained by positive genetic correlations between sexes, as the later have been recurrently recorded in studies on natural plant populations (e.g., Delph et al. 2002), as well as through artificial-selection experiments (Meagher, 1992; Delph *et al.*, 2004a,b, 2005, 2010; Scotti & Delph, 2006). In general, SD in gene expression is lower in plants than in animals, possibly reflecting the usually lower SD at the phenotypic level (Barrett & Hough, 2012). The low degree of SD may also reflect the usually incomplete separation of sexes observed in dioecious plant species. Indeed, the notion of gender in plants is often a quantitative trait in plants and not as discretely separated as it is in animals (Charlesworth & Charlesworth, 1981; Charnov, 1982). If the incomplete separation of sexes often observed in dioecious plant populations is adaptive, it may potentially limit the extent to which gender can become dimorphic in response to sex-specific selection, by maintaining sex-specific fitness optima closer to each other than would be expected in the case of a complete separation of sexual functions.

SBGE in vegetative tissues of natural dioecious populations of *Mercurialis annua*, characterized by balanced sex ratios, was investigated in Chapter 2 of this thesis. We observed that sexual dimorphism in gene expression is present from the earliest stages of seedling development, albeit involving only a low proportion of the total number of expressed genes. We hypothesised that SBGE in the vegetative phase of growth might in part reflect sex-specific developmental pathways

induced by sex-determining, potentially located on sex chromosomes. We failed to detect an accelerated rate of evolution for sex-biased genes, which showed no preferential sex-linkage, so that sexual conflicts might not explain sex-biased expression patterns in the species.

In Chapter 4, we reported results of an experimental-evolution study during which females of *M. annua* with frequent inconstancy in sex expression evolved higher levels of male allocation when deprived of male mates, in contrast with females in control populations maintained at a 1:1 sex-ratio. Interestingly, although there was a rapid increase in the mean pollen production by male-deprived females, the evolved populations continued to segregate 'canalized' female individuals that lacked a male function, suggesting, perhaps, a role of major genes in the expression of a male function in females. We refer to these two classes of females as 'phenotypically modified' and 'phenotypically canalized', respectively (or simply 'modified' and 'canalized'). Thus, while the phenotypically modified females in the male-deprived lines expressed the apparently selected increase in male function, the canalized females did not, even though their parents might have done. Although it is possible that the difference between modified and canalized females is an outcome of plasticity, it is nonetheless likely that parts of their genomes have been modified by selection over the previous generations of the experiment. These females may have undergone reduced levels of sexual selection, without the evolution of increased male allocation. The potential changes of sex-biased gene expression in these females may thus not be directly related to primary regulatory changes that have brought about increased male allocation, and might rather indicate a relaxation of SA selection. Our experimental evolution results thus provide fertile ground on which to investigate the evolution of sex-biased gene expression in case of a breakdown of dioecy, potentially also shedding light on the causes of the evolution of SBGE in *M. annua*.

The annual mercury clade contains functionally hermaphroditic lineages (more precisely, monoecious lineages) derived from dioecious ancestors. The breakdown of dioecy in these monoecious lineages is associated with differences in both geographical distribution and ploidy levels (Pannell *et al.*, 2008). Indeed, autotetraploid lineages of *M. annua*, found in southern Morrocco, are naturally monoecious. It is not known whether the breakdown of dioecy in *M. annua* was a coincidental outcome of whole genome duplication (e.g., caused by the disruption of gene expression related to gender separation), or whether selection for combined sexes over separate sexes led to a shift in the sexual system. Comparisons of the differences in gene expression between these natural monoecious lineages and females that have evolved a male function in our experiment should help us to understand both the long- and short-term implications of a particularly interesting case of the breakdown of dioecy.

In the present chapter, I report the transcriptomic consequences of selection on male allocation in females of *M. annua*. Our study aimed at comparing the transcriptional profiles of 'control' females with that of 'modified' and 'canalized' females from the selected lines of the selection experiment presented in Chapter 4. We analysed transcription profiles of leaf tissues from females and males of the experimental lines, as well as from individuals of the naturally occurring monoecious tetraploid lineage, grown in the same conditions. Finally we collected male flowers from males, modified females and monoecious individuals, in order to assess the similarity of expression between functionally identical tissues. More particularly, we were interested in the fate of genes previously identified as sex-biased (Chapter 2) and how their expression may change during evolution away from dioecy. We addressed the following questions: (i) Which genes are involved in the phenotypic changes observed in modified females of the selected lines? (ii) Is the predicted phenotypic masculinization of females of the selected lines underlined by expression changes in the sex-biased genes? (iii) Has the putatively relaxed sexual selection on females of the selected lines had an effect on sex-biased gene expression? (v) Are the transcriptional profiles of modified females drawing closer to naturally occurring monoecious *M. annua*, as might be expected?

Materials and methods

Plant material and phenotyping

To compare expression profiles of individuals from control and selected lines, we grew plants under controlled conditions during spring 2016 for eight weeks. For each selected line, we separated the 10 females with the highest male-flower production ('modified', or high-MRE females) and the 10 females with the lowest male-flower production ('canalized', or low-MRE females). We allowed this latter category to include females producing at most two male flowers. While canalized females were present in each selected line, we could sample only 5 such individuals in one of the replicates. From each control line, we sampled tissues from 10 males and 10 females, randomly chosen without regard to their male flower production. We recorded the number of male flowers, number and weight of seeds, plant height and above-ground biomass of each sampled plant. Mann-Whitney ranked tests were used to compare these variables between treatments. We simultaneously grew under the same conditions tetraploid hermaphrodite *M. annua* from three different natural populations in Morocco.

mRNA extraction, sequencing and measure of expression

After eight weeks of growth we sampled leaf tissues for all groups of diploid and tetraploid plants. We also collected male flowers from control males, modified selected females and tetraploids. RNA extractions were performed using the Maxwell® 16 Research Instrument, with the Maxwell® 16 LEV Plant RNA Kit. Library preparation was outsourced to Microsynth (using Nextera adapters). Sequencing was performed at the Center for Integrative Genomics (CIG, Lausanne), following the same protocol described in Chapter 2. Sequencing depth used for tetraploid samples was about twice that of diploid samples (Table S1). Expression levels were calculated based on a gene set of 34,006 predicted genes from the assembled genome of *M. annua*, using Kallisto (Bray *et al.*, 2016). Count matrices were used as input for differential expression, performed using DESeq2 (Love *et al.*, 2014), following the methods described in Chapter 2. As monoecious tetraploid populations are probably the result of autopolyploidization (i.e., not implicating hybridization; Obbard et al., 2006), we analysed gene expression in tetraploids using the same gene set, predicted from the *M. annua* diploid ancestor. The abundance in transcripts per million, scaled to the library size (resulting in *scaled(TPM))*, was performed with the tximport R package (Soneson *et al.*, 2016).

Analysis of gene expression

Differential expression between leaf and flower samples was analysed in DESeq2 (Love *et al.*, 2014). Normalization of abundance data was performed using DESeq2 for each of the three datasets independently: diploid leaf samples, diploid and tetraploid leaf samples, and flower samples. An adjusted p-value cut-off of 0.05 was applied for all analysis.

In order to investigate the potential masculinization or feminization of female transcriptomes during experimental evolution, we tracked changes in the expression levels of all expressed genes among which we previously identified 972 sex-biased genes (588 female-biased and 384 male-biased genes), in roots and leaf tissues (Chapter 2). We computed differences in the expression of genes between samples in leaf and flower tissues in log2(*ScaledTPM*) for each gene independently, using a ΔX approach (Zemp *et al.*, 2016), with $\Delta X = \text{mean}(X_{\text{sample A}}) - \text{mean}(X_{\text{sample B}}) / \text{s.d.}(X_{\text{sample A}})$. Functional enrichment of differentially expressed genes (DEG) was assessed using Fisher's exact tests, implemented in Blast2GO 3.0 (Conesa *et al.*, 2005), following methods described in Chapter 2.

Results

Phenotypic masculinization of the sex allocation of the selected females

The individuals from the fourth generation of selection, grown in glasshouse under controlled conditions, varied significantly for MRE (Mann-whitney rank tests, $P = 2.31 \times 10^{-6}$), with females from selected lines producing on average 129.9 times the number of male flowers produced by females in control lines (Figure 1d). No other morphological or allocation trait varied significantly between control and selected lines (Figure 1a-c). Within the selected lines, modified females produced an average of 63.2 (*SD* = 77.2) male flowers per individual, compared with canalized and control females, which produced an average of 0.4 (*SD* = 0.71) and 0.27 (*SD* = 0.58) male flowers, respectively.

Differential expression in leaves, across samples of the experimental populations

We found on average 27,486 expressed genes in leaf tissues of diploid individuals from control and selected lines. Expression patterns in leaf and flower tissues were substantially different (Figure 2). There was a particularly large amount of variation among diploid leaf samples (Figure 2). The differential expression we detected in leaves was higher among samples from different selection regimes than among samples from the same treatment, as indicated by the PCA plot representing the distribution of variance across our samples (Figure 2). Interestingly, the within-treatment variance in expression was greater among replicates of the selected lines than among those of the control lines (Figure 3). Under glasshouse conditions, sex-biased gene expression was low in control lines (in which there were only three DEG).

Comparisons between lines showed that canalized females of the control lines displayed the highest degree of differential expression with both males and females (287 and 133 DEG, respectively). Modified females from the selected lines revealed 172 and 121 DEG with control males and control females, respectively (Figure 4). These later DEG were significantly enriched for functions related to the regulation of gene expression and cellular nitrogen metabolism (Table 1). Sixty-five genes were similarly differentially expressed between females of the selected lines and females of the control lines. Finally modified and canalized females displayed 17 DEG for which we didn’t find any significant enrichment for a particular gene function.

Evolution of sex-biased gene expression in leaves during the selection experiment

We investigated the change in expression level of previously identified sex-biased genes (SBG) (Chapter 2) between our different leaf samples (Figure 5). Within the control lines, males and females differed significantly with respect to the expression of sex-biased genes, with higher

expression of male-biased genes in males and higher expression of female-biased genes in females, as expected. Males in the control lines had a higher expression level of male-biased gene expression compared to all other samples in the experiment. Females of the control lines displayed a higher level of expression of female-biased genes compared to all other samples, except canalized females. Within the selected lines, canalized and modified females differed significantly in their level of expression of SBG, with a higher expression of female-biased genes (Mann-Whitney rank tests $P < 2.2 \times 10^{-16}$), and lower expression of male-biased genes in canalized females (Mann-Whitney rank tests, $P = 1.17 \times 10^{-4}$), compared to modified females. In comparison non-biased genes displayed a null median of change of expression between these samples.

Comparisons between samples of control and selected lines showed that modified females have evolved an increased expression of male-biased genes, while female-biased genes showed reduced expression (Figure 6, Table 3). Female-biased genes showed greater variance in the change of expression (var = 0.60) than male-biased and non-biased genes (var = 0.52 and 0.46, respectively). Non-biased genes also showed an overall significant increase of expression in modified females compared to control females. Conversely, canalized females had similar level of expression of sex-biased and non-biased genes than did control females, with the exception of a slight but significant increase in male-biased genes (Figure 6a, Table 3). Comparisons with control males revealed patterns of gene expression changes in canalized females similar to differences observed between control females and males (Figure 5). In contrast, modified females displayed lower expression of both male- and female-biased genes in comparison to males from the control lines.

Comparisons of sex-biased expression in leaves, with monoecious tetraploids

In tetraploids, there were 27,454 and 28,555 genes expressed in leaf and flower samples, respectively. Gene expression varied more between leaves of different ploidy levels than between sexes of the same ploidy (Figure 7). Tetraploids displayed a lower level of male-biased gene expression compared to modified females and males. In contrast, females with low MRE (canalized females and control females) had lower male-biased gene expression than tetraploids. Patterns for female-biased genes were exactly the opposite, except that tetraploids had a lower level of expression of female-biased genes than did males. Interestingly, the differences in expression level of sex-biased genes were less important between tetraploid and modified females than they were between tetraploids and either canalized or control females (Figure 8). In all comparisons, tetraploid individuals displayed an overall lower expression level of non-biased genes (Figure 5).

Comparisons of expression in male flowers

Among our samples, three categories produced male flowers in sufficient quantity to analyse their gene expression: control males, modified females and monoecious tetraploids. Male flowers expressed 28,920 genes on average across samples, and their profile of gene expression contrasted substantially with that of leaf tissues, irrespective of the ploidy level (Figure 9). These tissues showed a higher degree of differential expression than leaves: we found 2,607 DEG (c.a., 9.33 % of expressed genes) between control males and modified females. When comparing flower tissues from tetraploid individuals, we detected a substantial number of SBG between tetraploid and modified females (9,447 genes), as well as between tetraploids and males (11,293 genes). The former DEG showed significant enrichment for various processes, including cellular nitrogen metabolism (Table 6), the later being significantly enriched for functions associated with plastidial and ribosomal activities (Table 7). In both cases, genes related to endonuclease activity were under-represented in these comparisons. Taking advantage of the dataset produced in chapter 2, we were able to estimate a neutrality index (NI) for the DEG detected in male flowers (McDonald & Kreitman, 1991). There was 155 male-biased genes, 256 ‘modified’-female biased genes and 4093 non-biased genes among the orthologs previously inferred with the closely related species *M. huetii.* Genes that were more expressed in modified females had a significantly lower NI estimates ($P = 0.0032$) compared to non-biased genes, while male-biased genes didn’t show any difference.

Sex-biased genes previously identified in vegetative tissues also showed changes of expression in male flower tissues. Male-biased genes had a significantly higher expression in male flowers from males compared to the other samples (Figure 10), while they displayed lower female-biased gene expression compared to the other samples.

Discussion

Gene expression changes between control and selected lines

The selection experiment conducted over four generations in male-deprived populations resulted in strong directional and disruptive selection on male reproductive effort (MRE) (Chapter 3). In response to selection, the average MRE of females in these lines increased, reaching high level of male allocation in some individuals while others, we termed ‘canalized’ females, produced only fruits and seeds. The selection regime had a strong influence on gene expression not directly related to male allocation, as canalized females and control females displayed the highest level of differential expression despite similar MRE. It is likely that most of the differential expression detected between modified females and control females was similarly not directly related to the response to selection on MRE we observed at the phenotypic level.

The increased male allocation in modified females, which was likely driven by selection for reproductive assurance and opportunities for outcrossing siring success in the absence of competitive males, must ultimately reflect primary regulatory changes in the transcriptome. Accordingly, we observed in this study that the increase in MRE of modified females was specifically accompanied by an increased expression of male-biased genes, a lower expression of female-biased genes, and a moderate increase in the expression of non-biased genes on average across genes and samples.

Regulatory changes in sex allocation are known to be controlled by both autosomal and sex-linked loci that tend to be physically linked in quantitative trait loci (QTL), in a number of plant species (Delph *et al.*, 2010; Spigler *et al.*, 2011). Most of the sex-biased genes that we inferred from vegetative tissues in Chapter 2 and used as a basis for the analysis presented in the current chapter, are autosomal. The present results show that their up- or down-regulation clearly does not require a Y-chromosome, which was of course entirely absent from the selected lines. Gene expression is known to be, at least in part, controlled by hormones in plants (Golenberg & West, 2013), so that changes in sex-biased gene expression may not need sex-chromosome when hormone concentration can be altered by autosomal loci. Indeed exogenous application of hormones such as auxins or cytokinins are known to alter sex expression in *M. annua* (Durand & Durand, 1991). However this pattern contrasts with results from previous studies on *Silene latifolia* (Scotti & Delph, 2006; Zemp *et al.*, 2016), or *Fragaria virginiana* (Spigler *et al.*, 2011), where sex-chromosomes evidently harboured an excess of QTL responsible for sexually dimorphic traits. These species differ in many aspects from *M. annua*, some of which may explain the discrepancy in our results. Both *S. latifolia* and *F. virginiana* have likely evolved dioecy via the gynodioecy-pathway, so that they both likely carry sterility mutations that play a key role in sex determination (Lardon *et al.*, 1999; Spigler *et al.*, 2008). The presence of such mutations in a linkage group that subsequently accumulated loci that modify male expression in hermaphrodites is thought to favour the suppression recombination in the SDR and may as well accompany responses to selection for biased expression at other loci to balance the potentially associated negative pleiotropic effects of sterility. In contrast, *M. annua* is more likely to have recently evolved dioecy through a monoecious intermediate step, so that sex allocation of males and females may have undergone gradual specialization. This process is likely slower than that involving the spread of sterility mutations (Charlesworth & Charlesworth, 1978b), so that the intensity of sexual conflicts might be reduced, too. *Fragaria virginiana* is an octoplyploid lineage carrying homologous ZW proto-sex-chomosomes, while *Silene latifolia* carries a highly differentiated Y-chromosome. Conversely, *M. annua* carries homomrphic XY sex

chromosomes that are only slightly differentiated (Ridout *et al*., bioRxiv). Our results may shed new light on a putatively minor role for sex chromosomes in driving SBGE in dioecious species that have evolved from a monoecious ancestor.

Changes in sex-biased gene expression in canalized females were limited compared to modified females. Indeed, we detected a slight but significant increase in the expression of male-biased genes in canalized females compared to control females, possibly driven by genetic correlation with modified females in the population. Modified-females were actually selected for increased maleness in the selected lines, possibly creating the same type of sexual conflicts present between gender in natural population. Under such conditions canalized females may undergo similar levels of sexual antagonism as in natural population, explaining why sex-biased gene expression satyed similar to that of females in the control lines.

Second, our result may indicate that female-biased genes are not necessarily kept at a sub-optimal expression level in natural populations of *M. annua*, as a result of unresolved SA selection, as was inferred for *Drosphila melanogaster* (Hollis *et al.*, 2014). However, it is interesting to note that the expression changes of sex-biased genes that we observed in modified females compared to control females concerned only 68.3% and 69.4% of female- and male-biased genes, respectively. No change, or indeed a shift in the other direction, was observed to characterize the rest of the sex-biased genes. These genes may not be directly involved in male allocation or related function, and may have evolved sex-bias under other pressure than primary regulatory changes associated with sex allocation.

The decreased expression of female-biased genes associated with the increased male allocation of modified females may reveal pleiotropic effects. These effects may as well be mostly attributable to primary regulation of sex allocation. Indeed, sex-biased genes in our dataset contained genes known to be involved in the transition of meristems to flowering, some of which were female-biased (see Chapter 2). The shift of sex allocation most likely changed the expression of genes involved in floral development, which may have resulted in a decrease in female-biased genes. However, this hypothesis is difficult to assess and would require functional analysis of gene expression.

Comparison of expression profiles with monoecious tetraploids

Most of the observed differences in sex-biased gene expression between control males and females and naturally occurring monoecious individuals involved male-biased genes. In particular, male-biased genes were more expressed in males, while female-biased genes were more expressed in

females, as expected for specialized unisexuals. Interestingly, while control females expressed more female-biased genes than did tetraploids, as expected, this was also so for males. This result tends to indicate a particular down-regulation of female-biased genes in tetraploids. Modified females showed a similar pattern, although the expression of female-biased genes in these females was even lower than in monoecious individuals. Like modified females, monoecious *M. annua* are most likely derived from a female form of the diploid lineage, as tetraploid individuals lack a Y-chromosome (Russell & Pannell, 2015). The important increase of expression of male-biased genes may indicate selection for increased male allocation, similar to the case of modified females. The lower divergence of expression profiles between these individuals points in the same direction. However, monoecious individuals displayed lower expression of female-biased genes than did either males or females. This result might point to an outcome of pleiotropy associated with the up-regulation of male-biased genes, or it may indicate sexual conflicts in the monoecious lineage that lead to a decreased expression of genes that are advantageous for the female function. Nonetheless, these speculations do not of course take into account the whole-genome duplication event associated with monoecy, nor its possible effects on gene regulation and rearrangements of gene networks (Buggs, 2013).

Comparisons of expression in flower tissues

Flower tissues were characterized by a strong divergence in expression between the three pollen-producing samples, resulting in high differential expression. Many differences may arise from the differences associated with the increased ploidy of monoecious individuals, as stated for leaf tissues above. However, it seems that these tissues have contrasted expression profiles between diploid males and modified females. Although functionally similar, we occasionally observed phenotypic variation of male flowers in modified females. Indeed, and as reported previously by Yampolsky (1919), modified females sometimes produced male flowers with different levels of intergradations between male and female functions, with what appeared to be initiated carpels present in male structures. If the flower samples in our experiment included such flowers (which were sampled as unopened buds), some of the variation in gene expression we observed might relate to the phenotypic intergradations.

Sex-biased gene expression, in particular for male-biased genes, displayed important expression differences between flowers of males and modified females. Once more, this result may be due to the overall higher male allocation of males, and thus may be a result of primary regulatory shifts in male flowers. It is possible that high expression of male-biased genes, in contrast to what we observed in leaf tissues, are caused by sexual conflicts in the dioecious natural populations. Indeed,

in dioecious populations, male flowers are functionally male-specific, and it is unlikely that low axillary pollen produced by inconstant females may sire many seeds in these populations (Chapter 3). As a consequence, gene expression may have undergone sexual specialization in males, potentially as a result of adaptation to their male-specific pedunculate infloresence, or driven by sexual selection through male-male competition. In *Arabidopsis thaliana*, for instance, genes related to pollen growth and pollen tube development showed typically biased expression, with increased protein evolutionary rates, consistent with patterns of sexual selection in flowers (Gossmann *et al.*, 2013). Our measure of neutrality index on genes differentially expressed in this tissue between modified females and males showed an increased level of directional selection (NI < 1) compared to the rest of the genome. On the contrary most sex-biased genes inferred from vegetative tissues showed no difference in evolutionary rates compared to non-biased genes. This may highlight sexual specialization on genes involved in flower production in *M. annua*.

Concluding remarks

Overall, our investigation of expression profiles of sex-biased genes during an experimental evolutionary transition from dioecy to monoecy has revealed a quantitative shift of sex-biased gene expression in leaf tissues, associated with the transition. The observed patterns suggests that SBGE in dioecious populations may be directly caused by primary regulatory changes rather than by the resolution of sexual conflicts within the genome. We also detected changes in SBGE not directly associated with a phenotypic change of sex allocation, which suggests that a minority of sex-biased genes may nonetheless have evolved differential expression to reduce sexual conflicts that are unresolved in the dioecious population. The transcriptome comparison between modified female and natural monoecious tetraploid individuals showed reduced differences in terms of SBGE, possibly confirming that the tetraploid lineage originated from genome duplication in a female, and that part of their balanced sex allocation has been driven by selection for reproductive assurance or selection for siring success in the absence of competitive males.

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FIGURES

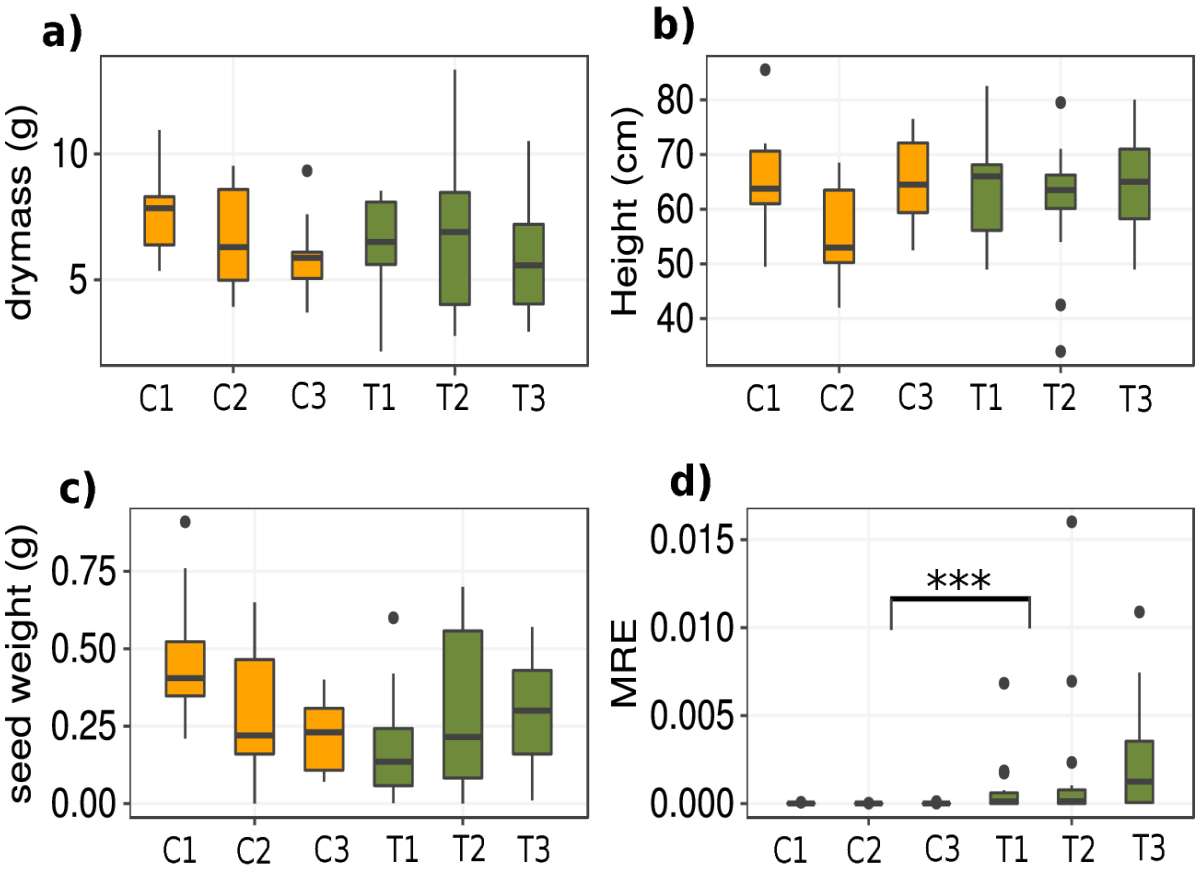

Figure 1. Phenotypic differences between control (orange) and selected (green) females, in **(a)** above-ground drymass, **(b)** height, **(c)** seed weight and **(d)** male reproductive effort (MRE). Whiskers represent standard deviation. Stars indicate significant difference between control and selected lines (male reproductive effort were compared using Mann-Whiney ranked tests, the other variables using Student's *t*-tests), *** : $P < 0.001$.

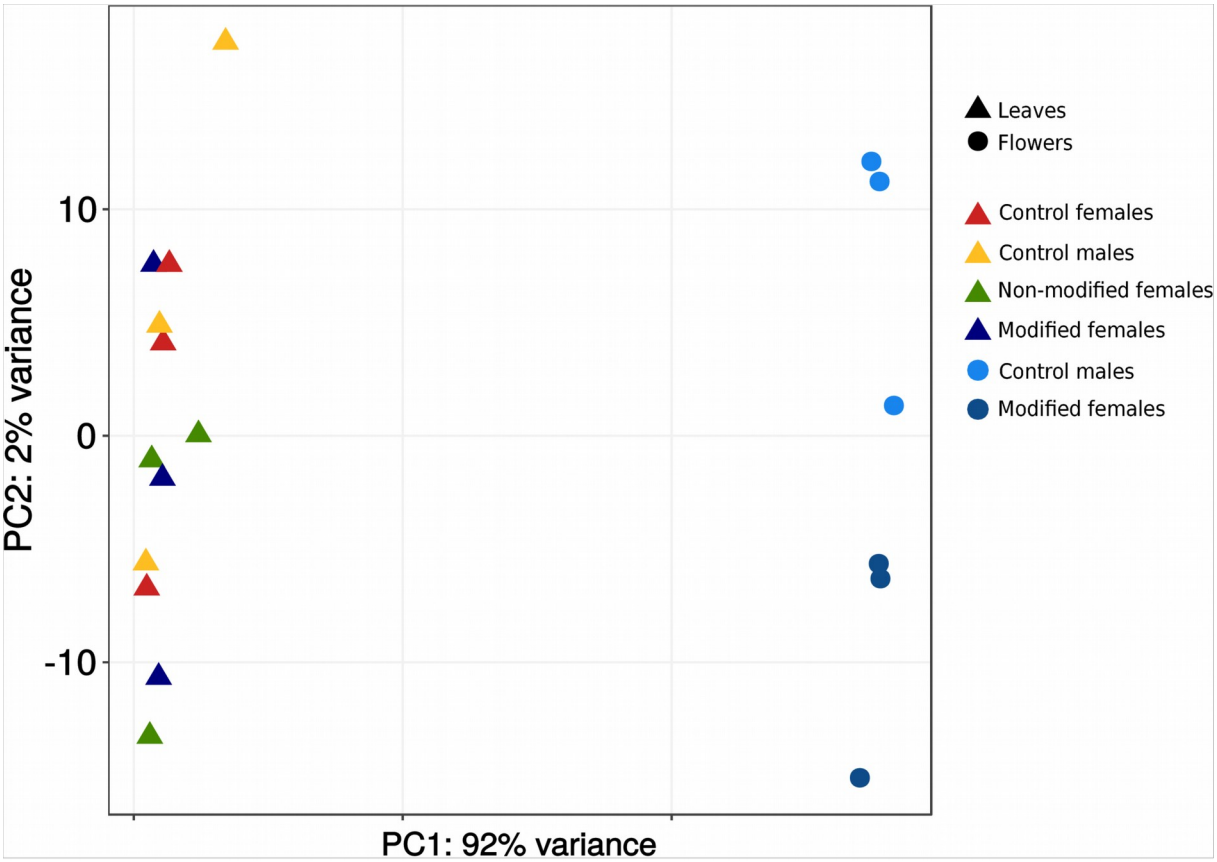

Figure 2. PCA plot of all diploid RNA samples grown in greenhouse. Circles represent flower samples, triangles represent leaf samples.

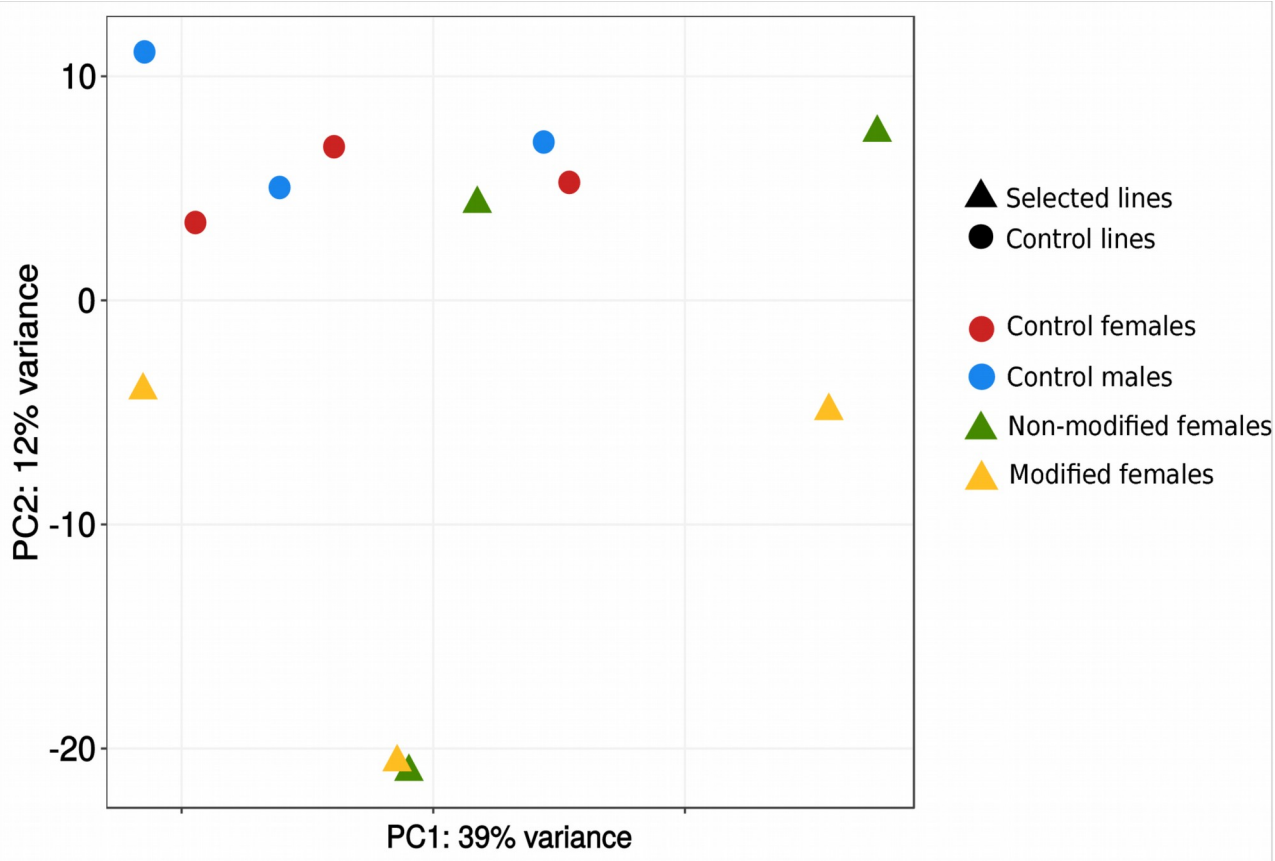

Figure 3. PCA plot of all diploid leaf samples grown in greenhouse. Circles represent flower samples, triangles represent leaf samples.

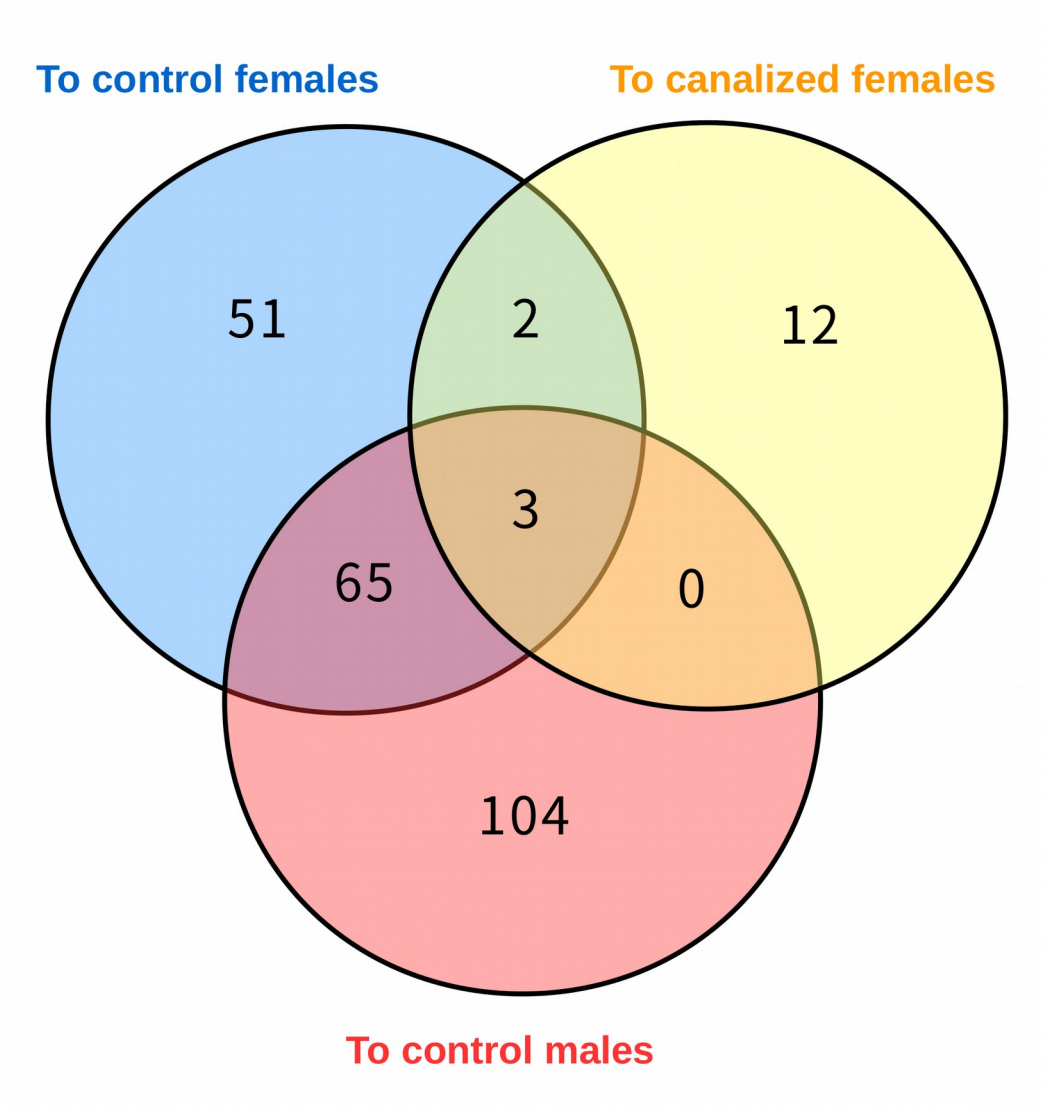

Figure 4. Overlapping patterns of the number of differentially expressed genes (DEG) between modified females and control females (blue), unmodified females (yellow) and control males (red).

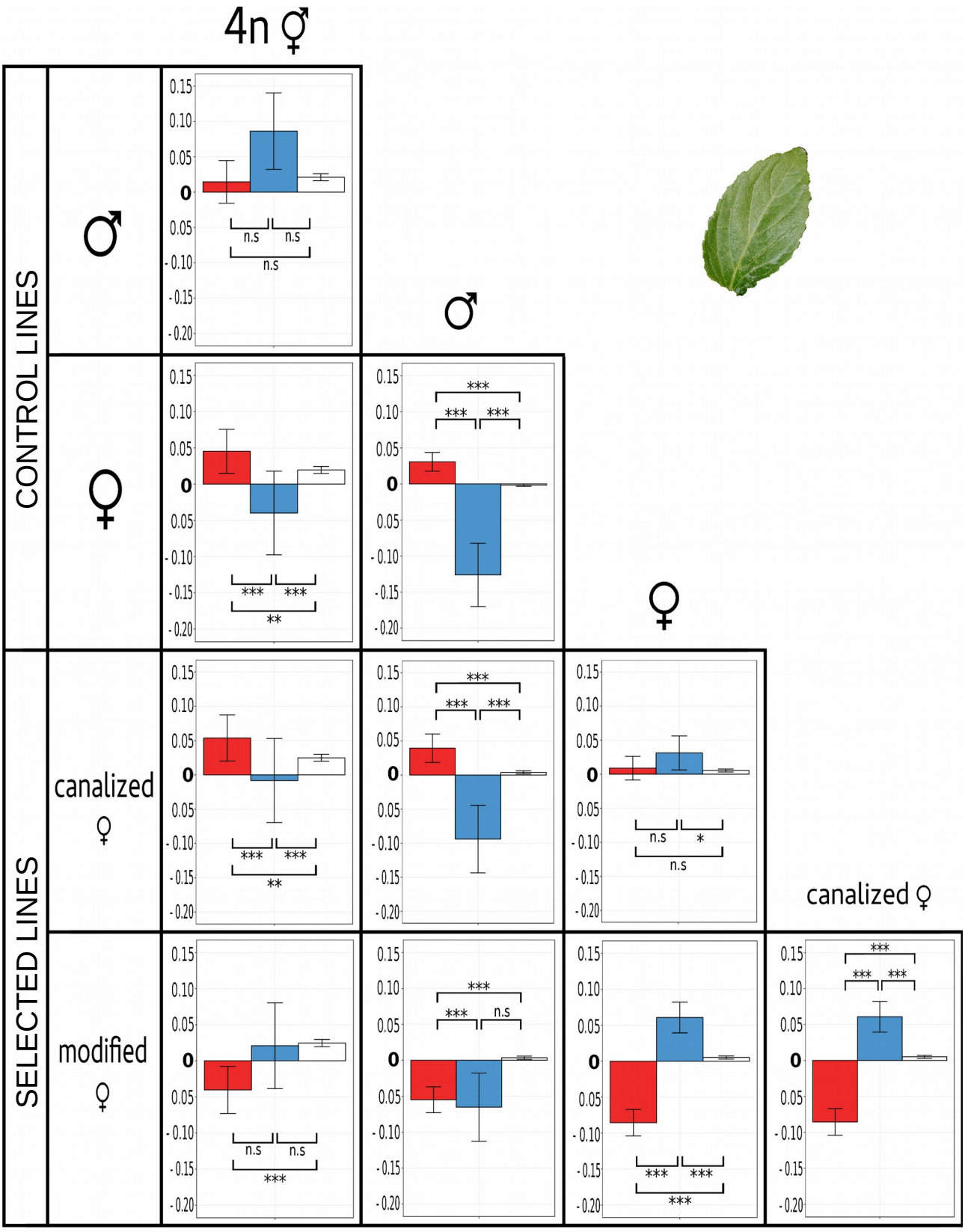

Figure 5. Expression changes (*ΔX*) in female-biased (red), male-biased (blue) and non-biased genes (white), between pair of leaf samples of *M. annua*. Positive values indicate higher expression in samples shown in the left-hand columns (sample A in the *ΔX* calculation, see Materials and Methods). Median are shown with 95% confidence intervals, of expression differences in $\log_2$(*scaled-TPM)*.

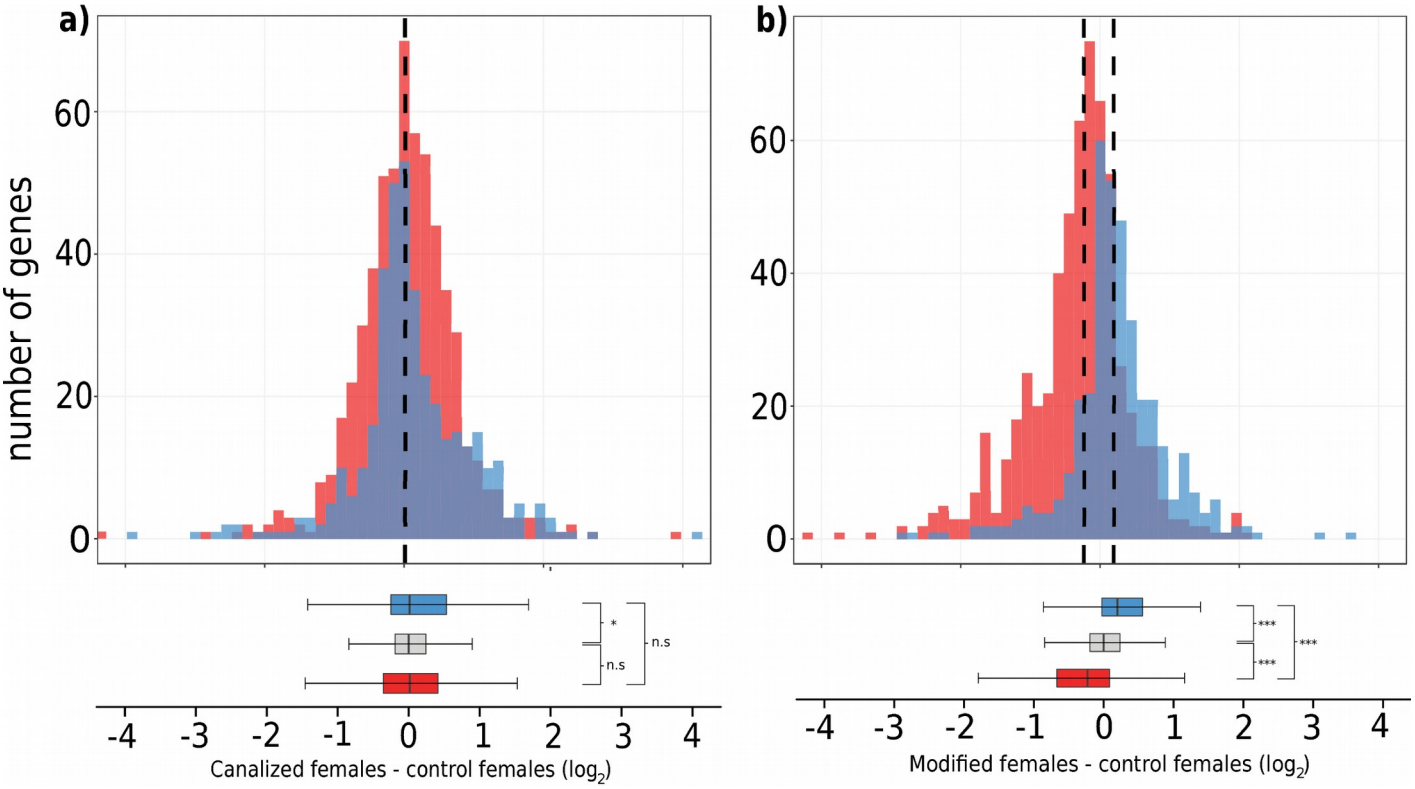

Figure 6. Change of sex-biased gene expression under pollen limitation in leaf tissues. Mean change of expression **(a)** in canalized females and **(b)** in modified females, compared to control females. Positive values indicate lower expression in control females. Stars indicate significant differences between gene sets, based on Mann-Whitney tests , * : $0.01 < P < 0.05$; *** : $P < 0.001$. Dashed lines represent median for female-biased and male-biased genes.

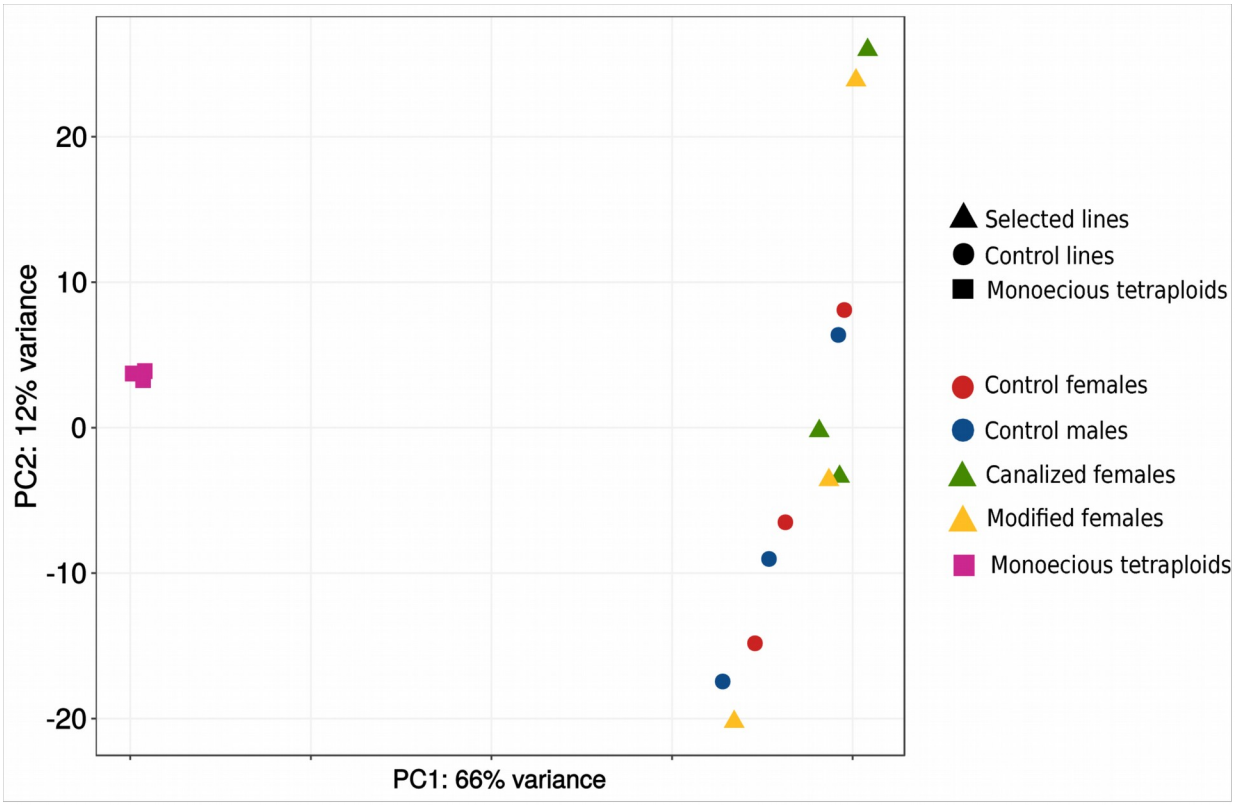

Figure 7. PCA plot of all diploid and tetraploid leaf samples grown in greenhouse.

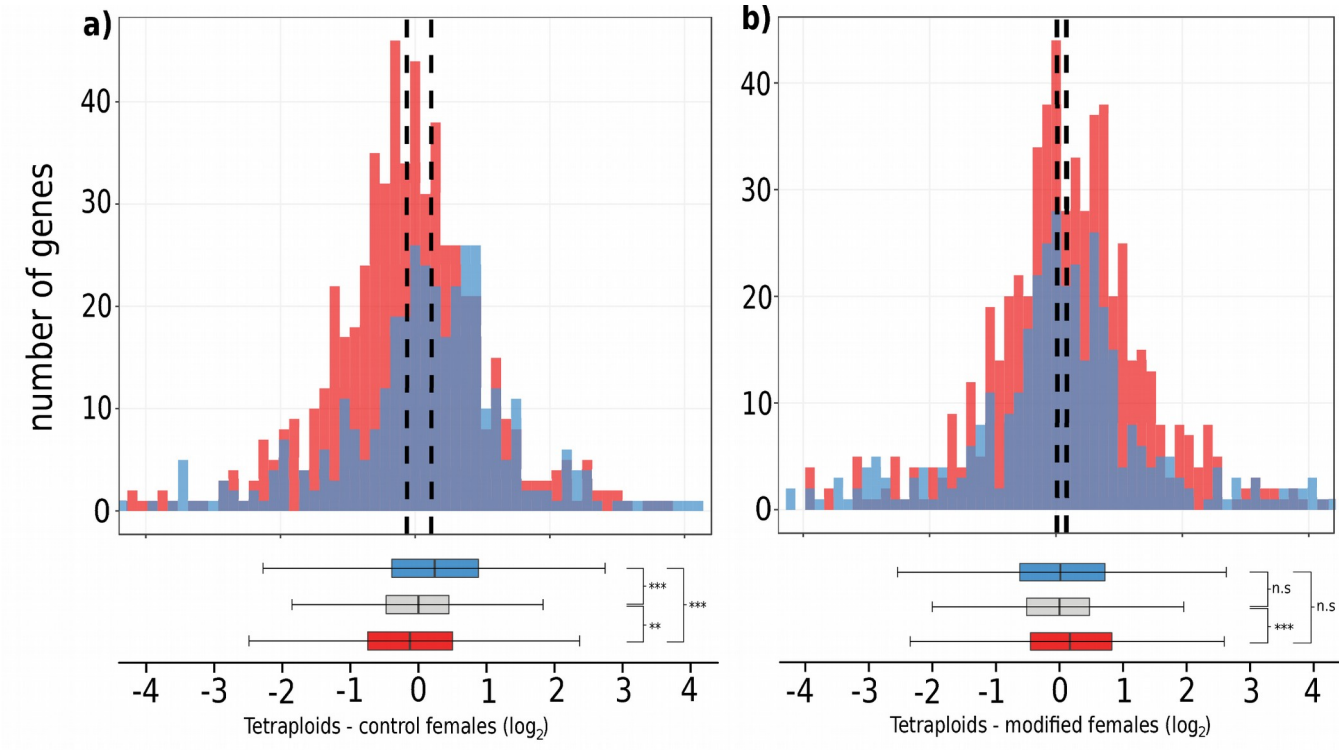

Figure 8. Difference of sex-biased genes expression in leaf tissues of tetraploid *M. annua*, compared to **(a)** control females and **(b)** modified females. Positive values indicate higher expression in tetraploids. Stars indicate significant differences between gene sets, based on Mann-Whitney tests, * *:* $0.01 < P < 0.05$ *;* ** : $0.001 < P < 0.01$ *;* *** : $P < 0.001$. Dashed line represent median for female-biased and male-biased genes.

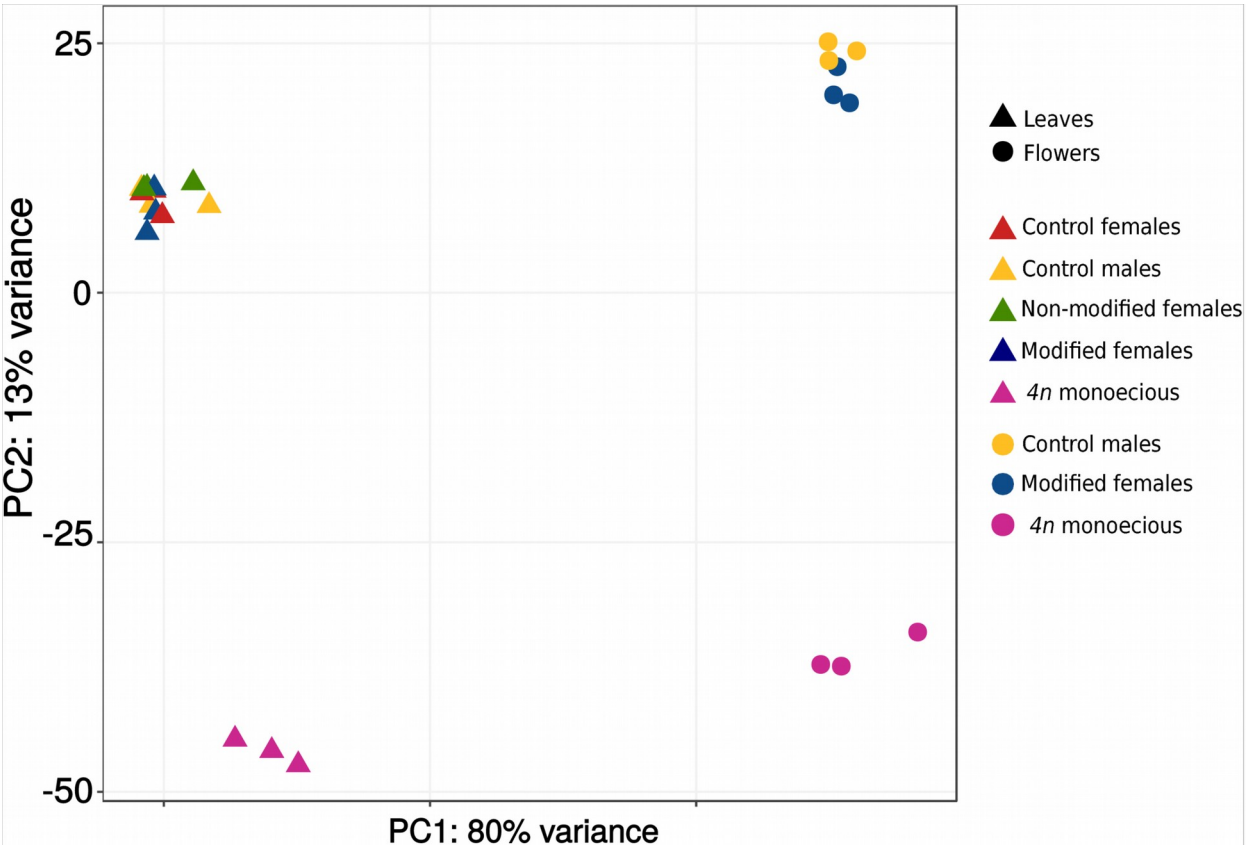

Figure 9. PCA plot of all RNA samples grown in greenhouse. Circles represent flower samples, triangles represent leaf samples.

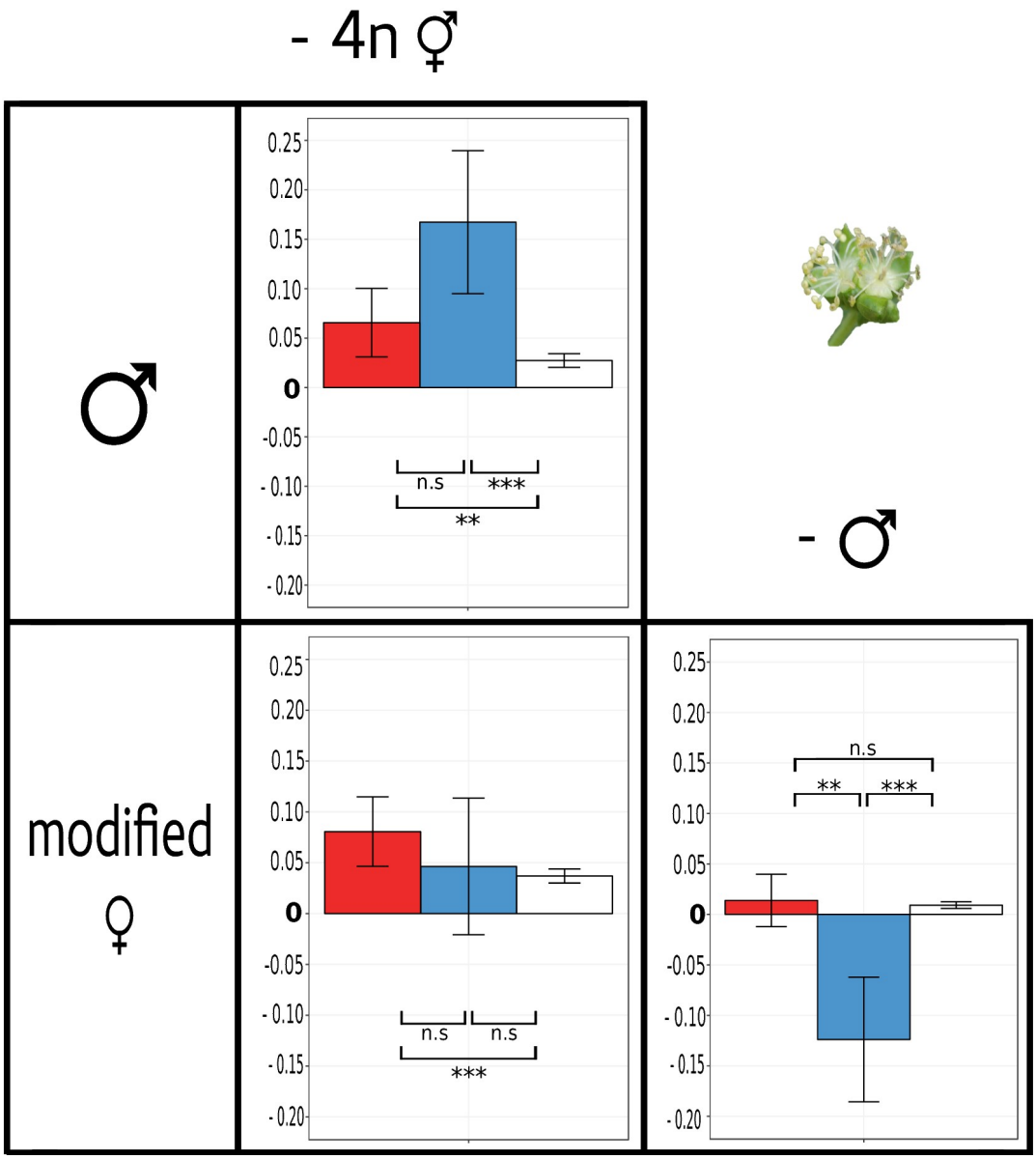

Figure 10. Expression changes in female-biased (red), male-biased (blue) and non-biased genes (white), between pair of flower samples of *M. annua*. Positive values indicate higher expression in samples shown in the left-hand columns. Median, with 95% confidence intervals, of expression differences in $\log_2$(*scaled-TPM).*

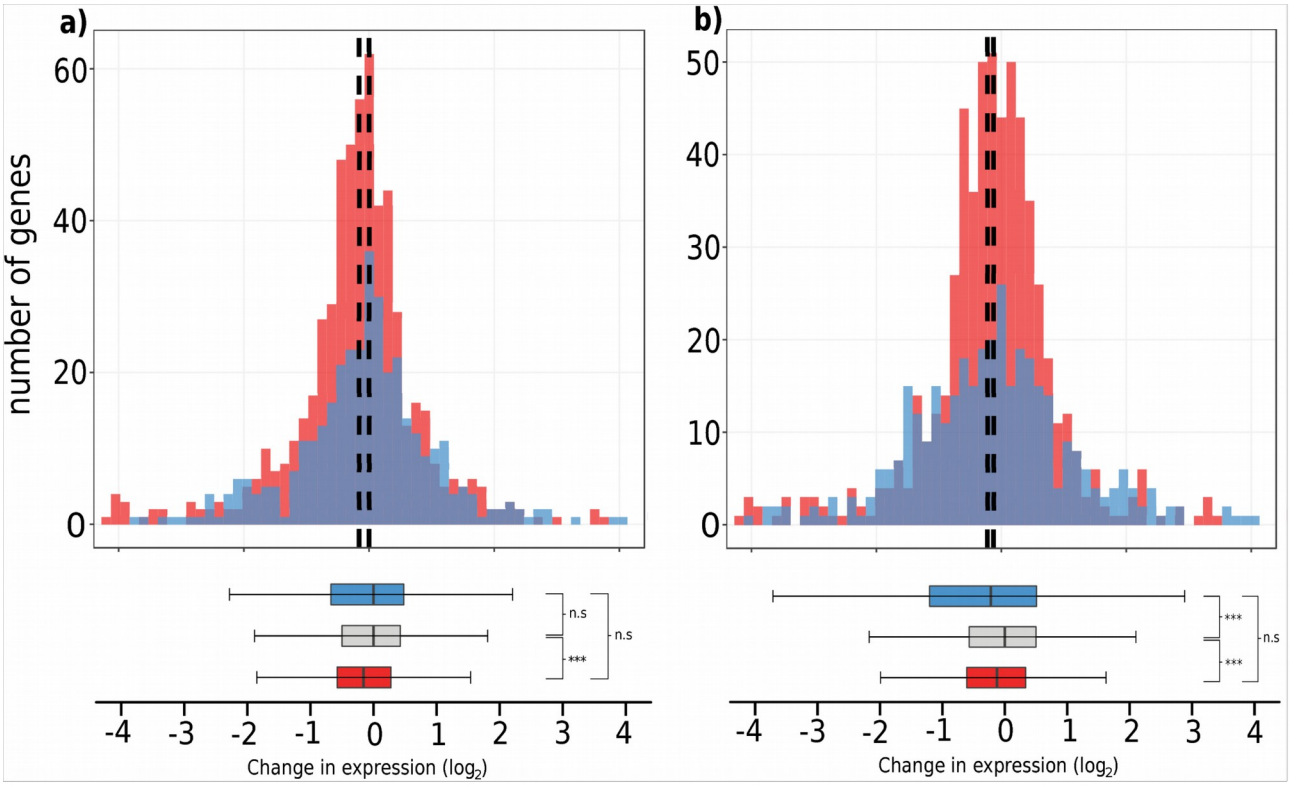

Figure 11. Change in sex-biased gene expression in *M. annua* for male flower tissues, **(a)** in tetraploids compared to modified females, and **(b)** in tetraploids compared with control males. Positive values indicate higher expression in tetraploids. Stars indicate significant differences between gene sets, based on Mann-Whitney tests. * : $0.01 < P < 0.05$; ** : $0.001 < P < 0.01$; *** : $P < 0.001$. Dashed lines represent median for female-biased and male-biased genes.

TABLES

Table 1. Fisher's exact tests for GO-terms enrichment (FDR < 0.05) of significantly over-represented gene functions among DEG between modified selected females and control females of *M. annua* (eight representative terms presented). No under-represented gene function was detected.

GO-ID	Term	FDR
GO:0003735	structural constituent of ribosome	1.10×10^{-24}
GO:1990904	ribonucleoprotein complex	2.85×10^{-23}
GO:0043228	non-membrane-bounded organelle	1.43×10^{-17}
GO:0006412	translation	6.47×10^{-16}
GO:1901566	organonitrogen compound biosynthetic process	1.91×10^{-16}
GO:0032991	macromolecular complex	4.25×10^{-13}
GO:0044271	cellular nitrogen compound biosynthetic process	1.29×10^{-9}
GO:0010467	gene expression	2.51×10^{-7}

Table 2. Fisher's exact tests for GO-terms enrichment (FDR < 0.05) of differentially expressed genes in male flower tissue between control males and modified females of *M. annua* from the selection experiment, in male flower tissue. Eight representative terms are presented.

GO-ID	Term	Over/Under represented among DEG	FDR
GO:0009579	thylakoid	Over	2.02×10^{-26}
GO:0019682	glyceraldehyde-3-phosphate metabolic process	Over	5.00×10^{-23}
GO:0042254	ribosome biogenesis	Over	1.66×10^{-21}
GO:0016072	rRNA metabolic process	Over	2.15×10^{-18}
GO:0051156	glucose 6-phosphate metabolic process	Over	5.66×10^{-17}
GO:0008270	zinc ion binding	Under	2.23×10^{-6}
GO:0004519	endonuclease activity	Under	7.07×10^{-5}
GO:0004523	RNA-DNA hybrid ribonuclease activity	Under	1.66×10^{-4}

Table 3. *P-values* of Mann-Whitney tests comparing the difference of expression level of leaf tissues, in $\log_2$(*scaled-TPM)*, of male-biased, female-biased and unbiased genes of *M. annua* showing **(a)** comparisons between canalized selected females and control females and **(b)** comparisons between modified females and control females. * : $0.01 < P < 0.05$; ** : $0.001 < P < 0.01$; *** : $P < 0.001$.

a)

$\log_2$(scaled-TPM)	**Unbiased**	**Female-biased**	**Median**
Unbiased			0.00
Female-biased	0.61		0.014
Male-biased	0.042 *	0.11	0.013

b)

$\log_2$(scaled-TPM)	**Unbiased**	**Female-biased**	**Median**
Unbiased			0.00
Female-biased	$< 2.2 \times 10^{-16}$ ***		-0.23
Male-biased	$< 2.2 \times 10^{-16}$ ***	$< 2.2 \times 10^{-16}$ ***	0.20

Table 4. *P-values* of Mann-Whitney tests comparing the difference of expression level of leaf tissues of *M. annua*, in $\log_2$(*scaled-TPM)* of male-biased, female-biased and unbiased genes, **(a)** between control females and tetraploids; **(b)** between modified females and tetraploids. * : $0.01 < P < 0.05$; ** : $0.001 < P < 0.01$; *** : $P < 0.001$.

a)

$\log_2$(scaled-TPM)	**Unbiased**	**Female-biased**	**Median**
Unbiased			0.00
Female-biased	1.4×10^{-3} **		-0.12
Male-biased	2.3×10^{-6} ***	6.8×10^{-7} ***	0.24

b)

$\log_2$(scaled-TPM)	**Unbiased**	**Female-biased**	**Median**
Unbiased			0.00
Female-biased	1.4×10^{-6} ***		0.17
Male-biased	0.28	0.051	0.014

Table 5. *P-values* of Mann-Whitney rank tests comparing the difference of expression level in flower tissues, in log_2*(scaled-TPM)*, of male-biased, female-biased and unbiased genes of *M. annua*, showing **(a)** comparisons between tetraploids and modified females and **(b)** comparisons between tetraploids and control males. * : $0.01 < P < 0.05$; ** : $0.001 < P < 0.01$; *** : $P < 0.001$

a)

log_2(scaled-TPM)	**Unbiased**	**Female-biased**	**Median**
Unbiased			0.00
Female-biased	5.1×10^{-6} ***		-0.16
Male-biased	0.56	0.051	0.00

b)

log_2(scaled-TPM)	**Unbiased**	**Female-biased**	**Median**
Unbiased			0.00
Female-biased	8.2×10^{-4} ***		-0.13
Male-biased	1.7×10^{-5} ***	0.066	-0.22

Table 6. Fisher's exact tests for GO-terms enrichment (FDR < 0.05) of differentially expressed genes in male flower tissue between modified females and tetraploids of *M. annua*. Eight representative terms are presented.

GO-ID	**Term**	**Over/Under represented among DEG**	**FDR**
GO:0008152	metabolic process	Over	6.99×10^{-33}
GO:0009058	biosynthetic process	Over	2.49×10^{-7}
GO:1901564	organonitrogen compound metabolic process	Over	1.96×10^{-6}
GO:0043168	anion binding	Over	2.23×10^{-6}
GO:0004519	endonuclease activity	Under	3.16×10^{-6}
GO:0019538	protein metabolic process	Over	3.97×10^{-6}
GO:0004523	RNA-DNA hybrid ribonuclease activity	Over	5.65×10^{-6}
GO:0090305	nucleic acid phosphodiester bond hydrolysis	Under	3.79×10^{-5}

Table 7. Fisher's exact tests for GO-terms enrichment (FDR < 0.05) of differentially expressed genes in male flower tissue between control males and tetraploids of *M. annua*, in male flower tissue. Eight representative terms are presented.

GO-ID	**Term**	**Over/Under represented among DEG**	**FDR**
GO:0008152	metabolic process	Over	4.11×10^{-59}
GO:0010467	gene expression	Over	1.49×10^{-22}
GO:0043226	organelle	Over	8.64×10^{-22}
GO:1901566	organonitrogen compound biosynthetic process	Over	6.09×10^{-21}
GO:1901564	organonitrogen compound metabolic process	Over	1.19×10^{-19}
GO:0043604	amide biosynthetic process	Over	3.18×10^{-19}
GO:0006518	peptide metabolic process	Over	3.59×10^{-19}
GO:0004519	endonuclease activity	Under	4.68×10^{-13}

Table S1. Sequencing pair-of-reads counts of all sequenced *M. annua* pooled samples.

Sample	Replicate / Population	Ploidy	Gender	Tissue	Number of cleaned pair of reads (100bp)
L15	1015	4n	Hermaphrodite	Leaf	45,576,022
X15	1015	4n	Hermaphrodite	Male flowers	44,217,706
L19	1019	4n	Hermaphrodite	Leaf	41,947,909
X19	1019	4n	Hermaphrodite	Male flowers	37,175,282
L22	1022	4n	Hermaphrodite	Leaf	34,806,770
X22	1022	4n	Hermaphrodite	Male flowers	40,781,325
L7F	7	2n	Female	Leaf	24,780,412
L7M	7	2n	Male	Leaf	22,899,199
X7M	7	2n	Male	Male flowers	26,380,190
L1F	1	2n	Canalized Female	Leaf	31,112,806
L1M	1	2n	Modified Female	Leaf	59,385,497
X1M	1	2n	Modified Female	Male flowers	28,092,813
L2F	2	2n	Female	Leaf	23,383,875
L2M	2	2n	Male	Leaf	21,868,532
X2M	2	2n	Male	Male flowers	85,825,870
L3F	3	2n	Canalized Female	Leaf	45,492,654
L3M	3	2n	Modified Female	Leaf	33,754,789
X3M	3	2n	Modified Female	Male flowers	34,393,307
L5F	5	2n	Canalized Female	Leaf	33,724,614
L5M	5	2n	Modified Female	Leaf	40,138,255
X5M	5	2n	Modified Female	Male flowers	36,615,284
L8F	8	2n	Female	Leaf	47,414,400
L8M	8	2n	Male	Leaf	55,578,033
X8M	8	2n	Male	Male flowers	47,506,078

- CHAPTER 6 -

General discussion

In this thesis, I have attempted to characterize the selective forces that may play a role in transitions between separate and combined sexes in flowering plants. The astonishing diversity of sexual systems, and the large predominance of hermaphroditism among angiosperms, has led to the hypothesis of frequent transitions of sexual systems during angiosperm diversification (Renner, 2014). These transitions have recurrently been regarded as polarized – from a state of combined sexes within individuals and flowers (hermaphroditism) towards systems in which sexes are separated between individuals (dioecy). The evolution of dioecy from hermaphroditism has long been thought to be largely irreversible, and thus to be an 'evolutionary dead-end' (Bull & Charnov, 1985; Heilbuth, 2000; Barrett, 2013). As our understanding of plant evolution progresses, however, it has become clear that the breakdown of dioecy may have occurred frequently in nature, i.e., that it is frequently not a dead-end, although the mechanisms underlying such breakdown are still poorly understood. The widespread variation of sex allocation, frequently encountered in natural dioecious populations, provides an excellent source of variation upon which selection can act and trigger a shift towards combined sexes. By addressing several related questions concerning the way gender is manifest morphologically and at the level of gene expression in *Mercurialis annua*, a plant remarkable for the variation in sexual systems it presents, my thesis has thrown further light on a topic that has puzzled evolutionary biologists since Darwin (1877).

The first two main chapters of my thesis posed questions about the extent to which males and females differ from one another in terms of how they express their largely common genome (Chapter 2), and in how they ultimately express gender in terms of the production of male versus female flowers (Chapter 3). Chapter 2 revealed that subtle but important differences between the sexes in gene expression begin very early in a plant's life, channeling development towards one or other of the two sex-allocation extremes. Chapter 3 showed that, despite these differences between males and females, which we know are underpinned by a well-developed genetic sex-determining system (Russell & Pannell, 2015), both males and females show leaky gender expression, producing flowers of the opposite sex. The pattern I found in *M. annua* is unusual, because both males and females are inconstant in their sex expression; the common pattern is for only males to show such leakiness (Ehlers & Bataillon, 2007). Leakiness has long been thought to reflect evolutionary paths from hermaphroditism to dioecy, but it also may represent the starting point for reversions back to hermaphroditism. That possibility was the focus of Chapters 4 and 5. The selection experiment described in Chapter 4 demonstrated that selection for reproductive assurance can actually drive drastic changes in sex allocation that were quickly manifested in our experimental populations. Nonetheless the evolved phenotypes were not uniform across individuals of a population and some

continued to show canalized female phenotypes. Finally, in Chapter 5, we observed that most phenotypic changes of sex allocation that occurred during the selection experiment of Chapter 4 were paralleled by shifts in the same direction of sex-biased genes. We suggested accordingly that most observed sex-biased expression dioecious *M. annua* might reflect only primary regulatory changes rather than past or ongoing sexual conflicts.

In this last concluding chapter of my thesis, I first summarize in a little more detail the key results of each of the chapters, highlighting their importance and the extent to which they might influence the perspective we should take in understanding transitions between combined and separate sexes more generally. Finally, I point to a number of outstanding questions and perspectives for further research on sexual-system transitions and the way plants express their gender.

Main conclusions

A key finding of my thesis is that differences in regulation processes associated with sex-determination and sexual conflicts that may exist between sexes in the dioecious lineage of *M. annua* may result in low levels of sex-biased gene expression in several vegetative tissues. The expression profiles of the two sexes have thus diverged at only a few loci within the genome, while the great majority of genes were encoded similarly in males and females. The few sex-biased loci may be expressed early in the plant development, driving sexes apart, likely towards a sex-specific optimal strategy, and preventing them from expression functions of the opposite sex. This separation and independent optimization of sexes is constrained by their shared genome, which is perhaps reflected by the ability of both males and females occasionally to produce functional carpels or stamens, respectively, as has been observed in many dioecious plant species.

A key aim in my thesis was to investigate the evolutionary significance of labile sex expression, or inconstancy, for sexual system transitions. One point of significance was emphasized by (Lloyd, 1980), who showed how patterns of inconstancy and its distribution between the sexes might throw light on likely pathways that have been followed towards dioecy. When studying *M. annua*, we noticed that inconstancy was present in both sexes, although not symmetrically: males' inconstant investment represented a greater difference of allocation compared to the corresponding scenario for inconstant females. On the other hand, females were much more frequently inconstant than were males. These results indicated both that dioecious *M. annua* had likely evolved dioecy through a monoecious intermediate step, and that the breakdown of dioecy via selection on female inconstancy would be more likely than via selection on male inconstancy, in contrast to the path emphasized by published models (Ehlers & Bataillon, 2007; Crossman & Charlesworth, 2014).

In order to test the idea that female inconstancy could be a facilitator of sexual-system transitions away from dioecy, we established an experiment in which we removed males from experimental populations and compared the evolution of the remaining females with those that remained in experimental dioecious populations with the usual 1:1 sex ratios. This treatment subjected the females of the selected male-less lines under the influence of severe pollen limitation, immediately favouring those with inconstant sex expression. In these experimental lines, in which females could mate freely, we observed a rapid shift in the mean male allocation of females, even though many females continued to express a canalized female function only. The reversion we observed was gradual, accompanied by gradual changes in sex-biased gene expression.

After four generations of selection in the experimental populations, the evolved females resembled more the natural monoecious *M. annua*, in terms of expression profiles, than their original female form that was still observable in the control lines. Together, these results suggest that female inconstancy can indeed provide a starting condition for the breakdown of dioecy. This shift is directly influenced by the sex-ratio of the population, i.e., a decrease in male frequency triggers a shift towards increased male allocation in females, as was previously observed in the hexaploid *M. annua* lineage (Dorken & Pannell, 2009). It is thus possible that the European dioecious populations, characterized by frequent female inconstancy, are a particular case of androdioecy, in a metapopulation that contains only large sub-populations, allowing males to maintain at a high frequency, in turn driving the strongly female-biased sex allocation of females, and functional dioecy. The shift in sex allocation of females when the frequency of males is decreased is likely to be accompanied by changes in the mating system of the population, influenced by higher levels of selfing and the expression of inbreeding depression, for instance, which may counteract the benefits of reproductive assurance. We are currently assessing these processes to further understand the evolutionary trajectories undertaken by our experimental populations.

We remain ignorant of the genetic basis of sex-determination and sex inconstancy in *M. annua*, although our selection experiment probably points to the importance of many loci of small effect. Indeed, in Chapter 5, we speculated that the observed sex-biased genes were differentially expressed in dioecious *M. annua* because of primary regulatory changes associated with sex-determination, rather than as an outcome of sexual conflicts. Given the potential importance of sexual lability in evolutionary transitions between sexual systems in plants, an equally important field of investigation concerns the study of sex determination mechanisms and how, despite its role in maintaining two sexually distinct categories of individuals within population, it allows variation, i.e., inconstancy. We have seen in this thesis that the (monomorphic or dimorphic) pathway taken to

evolve dioecy likely influences patterns of inconstancy and the possibility of reversions towards functional hermaphroditism. It is possible that these pathways are also associated with different mechanisms of sex determination.

To some extent, our understanding of the genetic basis of sex-determination in plants is essential for a full understanding of the variation in sex expression we might observe in any given population. Although largely unknown in plants, recent progress has been made in understanding how sexes are determined and canalized along their sex-specific developmental pathways, and indeed how they can deviate from it. In particular there is an emerging view that sex-determination in plants is sometimes caused by a single locus, as supported by several recent empirical studies (Pucholt *et al.*, 2015; Akagi *et al.*, 2016; Chen *et al.*, 2016). This contrasts with the common 'two-loci' model of evolution of sex-determination in dioecious plants and might involve different mechanisms, potentially related to inconstant sex expression, like hormonal control of sex allocation for instance (Golenberg & West, 2013).

Further unanswered questions and perspectives

The breakdown of dioecy may potentially lead to the emergence of sexual systems different to the original sexual state from which dioecy has evolved in the first place. The results I presented in this thesis are mute on the irreversibility of sexual system transitions *per se*, defined by Bull and Charnov (1985) as "the inability of a population to reacquire a (recent) ancestral state" (Bull & Charnov, 1985). Indeed the reversibility of evolution supposes that an evolved population would reacquire the exact same character that was present before it diverged from its ancestors. In the case of sexual-system evolution, however, it is difficult to predict *a priori* whether the breakdown of dioecy should lead towards a sexual system similar to the ancestral state. For instance, dioecious species that have evolved through the dimorphic pathway involving sterility mutations (see Introduction) produce unisexual flowers structurally different from the bisexual flowers carried by their hermaphroditic ancestors, so that a breakdown towards monoecy cannot be ruled out for dioecious species that have evolved through a gynodioecious intermediate step. Indeed, closely related species can sometimes present different sexual systems, e.g., the Asteraceae contains gynodioecious, monoecious and dioecious species (Renner & Ricklefs, 1995; Torices *et al.*, 2011; Dufay *et al.*, 2014). This is particularly striking when we think about the emergence of androdioecy among angiosperms. This rare sexual system is unlikely to arise directly from hermaphroditism (Charlesworth & Charlesworth, 1978), and it seems to have occurred only on very few occasions under uncommon conditions (Dommée *et al.*, 1999; Gleiser & Verdú, 2005; Pannell & Verdú, 2006;

Gleiser *et al.*, 2008; Saumitou-Laprade *et al.*, 2010; Husse *et al.*, 2013; Billiard *et al.*, 2015). This system probably emerges more frequently through the breakdown of dioecy, which in turn likely evolved via alternative intermediate sexual systems (Pannell, 2000; Delph, 2009).

The investigation of the mechanism of sex determination and its potential relationships with the presence of sex chromosomes is necessary to understand transitions in sexual systems in *M. annua.* Along these lines, we have started the characterization of the Y chromosome in dioecious and androdioecious lineages. Specifically, we have sequenced the non-recombining region of the Y chromosome using a genome-walking approach with the sequencing, *via* PacBio, of BAC clones specific to this region. This has permitted us to identify a number of genes contained in the sex-determining region, and to assess how conserved they may be among lineages. In order to have a first estimate of the degree of degeneration of the Y chromosome, which is supposed to occur subsequent to the arrest of recombination of the SDR, we artificially produced individuals carrying two copies of the Y chromosome, by enforcing the mating between artificially feminized males with other males. Preliminary results indicate that YY males are viable and do not appear to display lower fertility than normal XY males. In order to study the consistence of sex-biased expression patterns among different ploidy levels and sexual systems in the annual mercury clade, I have sampled and sequenced many tissues, at different life stages, from the other lineages of the clade, in order to assemble transcriptomes and compare them to that of dioecious *M. annua*. In particular, the genus contains a dioecious allotetraploid species (*M. canariensis*) that can permit us to study the role that hybridization and whole-genome duplication may play in the outcome of sexual conflicts (Obbard *et al.*, 2006). The integration of data from perennial *Mercurialis* species would help in being more exhaustive, by integrating, for instance, monoecious diploid species such as *M. leiocarpa* (Krähenbühl *et al.*, 2002). It would also be informative to assess the role that plasticity might play in labile sex expression and in the breakdown of dioecy. A straightforward method to investigate this point would be to assess the amount of environmentally-induced variation in sex allocation, in other words the reaction norms of sex allocation, between an evolved and a control female from our selection experiment.

Finally, it seems that the male-specific pedunculate inflorescence has played an important role in transitions and maintenance of sexual systems in the *Mercurialis* genus, as discussed above. This trait is typically Y-linked and shows Mendelian inheritance. Uncovering the genetic basis of peduncle development would likely draw us closer to an understanding of sex-determination in *M. annua.* In particular, it would be interesting to know whether peduncles have emerged as a male-

beneficial trait that has facilitated the evolution of separate sexes, or whether this trait has emerged in populations that had already evolved dioecy by sexual specialization, perhaps preventing its reversion.

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