Lack of trade-offs between the male and female sexual functions in the gynodioecious herb *Geranium sylvaticum*

Sandra Varga[1] and Minna-Maarit Kytöviita[2]

[1]School of Life Sciences, University of Lincoln, Joseph Banks Laboratories, Lincoln LN6 7TS, UK

[2]Department of Biological and Environmental Science, University of Jyvaskyla, Jyvaskyla 40014, Finland

[1]Corresponding author: svarga@lincoln.ac.uk and sandravarga30@hotmail.com

Abstract

Resource allocation trade-offs between sexual functions are predicted, but these are usually measured only at the whole plant level. In some gynodioecious species, individuals with partially restored male fertility exist providing an opportunity to investigate whether the costs associated with male fertility restoration are linked to seed production. In this study, we examined whether there is a relationship between the number of stamens and seeds produced both at the flower and at plant level in the gynodioecious herb *Geranium sylvaticum*. We individually marked flowers in plants varying in the degree of male sterility in the field and counted the number of seeds produced. There was no significant correlation between the number of stamens and seeds produced at the flower level suggesting an absence of resource trade-off between female and male functions. Furthermore, number of stamens and seeds were positively correlated at the plant level. The lack of trade-offs between the two sexual functions in this species indicates that relative sex allocation was not affected by resources within a single season and/or that the male costs were relatively small.

Introduction

Plant resource allocation patterns have been fascinating evolutionary ecologists for decades. A basic assumption in resource allocation theory is that individuals possess a finite amount of resources (e.g. time or energy) to allocate to the different functions (growth, survival and reproduction). Individuals should allocate limited resources such that fitness is maximised (principle of allocation, Cody 1966; Levins 1968). Based on the idea of limited resources there should theoretically be trade-offs between resources allocated to different functions (Reznick 1985) and this has been empirically supported (Stearns 1989; Bazzaz and Grace 1997). Among all resource allocation decisions, resource investment in reproduction is expected to have the most important implications for the evolution of life history traits (Obeso 2002).

Most flowering plants are hermaphroditic, with flowers containing both the female (i.e. seed production) and the male (i.e. pollen production) sexual function. Sex allocation in hermaphroditic plant species usually departs from strict equisexuality (Ross 1990; Méndez and Traveset 2003). Trade-offs between allocation to female and male functions are generally considered significant and for instance one of the most important reasons behind sexual dimorphism and the evolution of dioecy (e.g. Ashman 1999). Even though sex allocation trade-offs have been investigated to some extent at the plant level, few studies have evaluated trade-offs at the flower level (but see Devlin 1989; Mossop et al. 1994; Sato and Yahara 1999). Yet trade-offs within an individual level are fundamental in modular organisms such as plants and may reveal phenomena that are not observable at the whole plant level. Species where hermaphrodite individuals vary in the number of anthers produced and thus vary in the degree of maleness provide an excellent and unusual opportunity to investigate allocation trade-offs between sexual functions.

Less than 1% of plant species are gynodioecious i.e. include populations where females coexist with hermaphrodites (Renner 2014; Rivkin et al. 2016). In most gynodioecious species, sex determination is cytonuclear, with cytoplasmic male sterility (CMS) genes disrupting pollen production, which is restored by nuclear genes (Schnable and Wise 1998). The outcome of the cytonuclear interaction depends on whether restorers have negative pleiotropic effects on fitness (Caruso et al. 2012). Even though theoretically demonstrated, few studies have quantified the pleiotropic costs due to the lack of available molecular markers for CMS (but see De Haan et al. 1997; Bailey 2002; Dufaÿ et al. 2008; del Castillo and Trujillo 2009). The restorers may have pleiotropic effects influencing both male and female fertility in hermaphrodites (Koelewijn 2003).

Geranium sylvaticum is a perennial species with a gynodioecious breeding system and its sex expression is most likely determined by the interaction between nuclear and cytoplasmic male-sterility genes (see Asikainen 2004; Couvet et al. 1998). Populations are typically comprised of female or 'male-sterile' individuals (i.e. plants with rudimentary, non-functional stamens) and individuals that produce pollen (i.e. hermaphrodites). Hermaphrodites possess flowers with one to ten functional stamens, so plants can be further classified as full hermaphrodites (i.e. producing only perfect flowers with 10 functional stamens), and intermediates (i.e. producing perfect flowers with one to nine functional stamens or with a mixture of pistillate and staminate flowers). The intermediate plants are probably the result of a partial male sterility restoration. In addition to the genetic mechanisms underlying male sterility restoration, biotic (Varga and Kytöviita 2010) and abiotic factors (Varga and Kytöviita 2016) may also affect the stamen number in *G. sylvaticum*.

Previously, we have shown that in *G. sylvaticum* populations the functional gender (sensu Lloyd 1980) of the hermaphrodite individuals varies and is biased towards maleness

(Varga and Kytöviita 2016). Theoretically, assuming a positive relationship between stamen number and paternity probability, there needs to be compensation for the fitness loss due to reduced stamen numbers to enable intermediates to coexist with full hermaphrodites, as both sexual phenotypes produce a similar amount of pollen per anther (Varga and Kytöviita 2014). It is usually assumed that a trade-off between female and male functions exists both at the flower and plant level and that allocation between the two sexual functions should also result in a strategy that maximises the sum of fitness gained through both functions (Parachnowitsch and Elle 2004; Ashman and Majetic 2006).

In this work, we investigate phenotypic sex allocation patterns in hermaphroditic individuals in a gynodioecious experimental population of *G. sylvaticum*. We assume trade-offs in resource allocation between female and male sexual functions at the flower level and consequently predict a negative correlation between seed number and stamen number both at the flower and at the plant level.

Material and methods

Study plant

Geranium sylvaticum (Geraniaceae) is a self-compatible, perennial plant. Most populations are gynodioecious, and individuals with an intermediate sexual expression have been reported, having similar or even higher frequencies than that of females in populations (Vaarama and Jääskeläinen 1967; Asikainen and Mutikainen 2003; Volkova et al. 2007).

Female flowers have rudimentary stamens and produce no pollen, whereas perfect flowers have one to ten functional stamens divided in two whorls of five and produce viable pollen. Pollen-producing flowers are protandrous and female flowers remain receptive for pollen longer than pollen-producing flowers (Varga et al. 2013a; Elzinga and Varga 2017). Flowers are visited by a large array of insect including syrphid flies, bees, and bumblebees.

All flowers offer nectar (Varga et al. 2013a). Higher visitation rates to hermaphrodite plants have been reported in some populations (Asikainen and Mutikainen 2005a), even though no study has investigated the patterns of visitation to individual flowers. All flowers produce a fixed number of ten ovules even though usually only up to five seeds develop per fruit (Asikainen and Mutikainen 2003).

Experimental plants

To examine trade-offs between female and male function we collected data in an experimental *G. sylvaticum* field located near Konnevesi Research Station, central Finland (62°38′N, 26°17′E). The field is an experimental setup design to evaluate long-term performance of *G. sylvaticum*. Briefly, the experimental plants originate from seeds obtained from a controlled hand-pollination experiment (Varga et al. 2013b). The experimental population is composed by 448 plants that germinated in early 2008 and were planted into the experimental field in September 2008 (see Varga et al. 2013b and Varga 2014 for more details).

Plant measurements

Flowering phenology of each individual was monitored every second day throughout the flowering period in 2010, noting the number of open flowers and the number of functional stamens in each open flower. During the peak flowering, we randomly marked more than 1000 flowers on 196 hermaphroditic plants. Individual flowers were marked with the number of functional stamens present using small transparent labels (5 x 5 mm): e.g. 0 for a pistillate flower with zero functional stamens; 10 for perfect flower with 10 functional stamens; 4 for an intermediate flower with four functional stamens. Plants were naturally pollinated by a range of insects including bees, bumblebees, syrphid flies and other diptera (pers. obs). After

seed production, flowering shoots were collected and all flowers, fruits and seeds were counted, noting the number of seeds produced on the marked fruits.

The sexual expression of each plant was calculated at the end of the flowering period as the average number of functional stamens present in all flowers observed (marked and unmarked flowers) per plant. Therefore, sexual expression of a plant ranged from 0 (all flowers produced zero functional stamens) to 10 (all flowers produced ten functional stamens), with intermediate plants with intermediate values.

Statistical analyses

All analyses were performed using R v.3.1.2. At the flower level, we first examined whether fruiting (Yes/No) was related to the number of stamens produced, using a generalised linear mixed model (GLMER) with a Binomial error distribution (package 'lme4') including plant identity as a random factor. At the flower level, we also investigated whether the number of seeds produced was related to the stamen number using a GLMER with a Poisson distribution and including the same variables as before.

At the plant level, we first investigated whether the number of shoots produced was affected by the plant sex expression using a generalised linear model (GLM) with a Poisson error distribution. Then, we tested whether the number of flowers, fruits and seeds produced were related to the sex expression of the plant and the total number of stamens produced per plant. For these, we used GLMs with negative binomial error distributions to correct for overdispersion (package 'MASS') including the number of shoots as a covariate.

In all models, residuals were visually inspected and collinearity between variables was evaluated calculating variance inflation factors (VIF), and in all cases VIF < 2.

Results

Individual flower level

Altogether, we retrieved data from 953 flowers belonging to 196 different individuals, with a mean ± SE number of stamens per flower of 7.6 ± 0.1 (Fig. 1). When analysed at the individual flower level, out of the 953 flowers marked 91% did successfully develop into a fruit containing at least one seed. The probability of a flower developing into a fruit was affected by the number of stamens present (ß = 0.01 ± 0.00, z = 12.0, p < 0.001). The marked flowers produced on average 2.90 ± 0.05 seeds per fruit. However, seed production at the flower level was not related to the number of stamens produced (ß = 0.01 ± 0.01, z = 0.71, p = 0.48; Fig. 2).

Whole plant level

The size of the experimental plants measured as the number of shoots ranged between one and four (average 1.4 shoots ± 0.1). The number of shoots was not related to the plant sexual expression (ß = 0.35 ± 0.35, z = 1.018, p = 0.308).

During the flowering season, plants produced on average 30.0 ± 1.4 flowers and this number was positively related to the number of shoots produced (ß = 0.34 ± 0.06, z = 6.206, p < 0.001) but not to the sexual expression of the plants (ß = 0.17 ± 0.22, z = 0.791, p = 0.429). On average, 17.3 ± 1.0 of the flowers turned into a fruit containing at least one seed. The number of fruits produced was independent of shoot number (ß = -0.05 ± 0.05, z = -0.955, p = 0.339), but was negatively related to plant sex expression (ß = -0.98 ± 0.19, z = -4.931, p < 0.001) and positively related to the total number of stamens per plant (ß = 0.04 ± 0.00, z = 16.199, p < 0.001).

Finally, plants produced on average 23.2 ± 1.2 seeds. We found a negative, significant relationship between seed production and plant sex expression (ß = -0.83 ± 0.29, z = -2.817, p < 0.01; Fig. 3). Moreover, the number of seeds was affected by the total number of stamens

produced at the plant level (0.04 ± 0.00, $z = 11.295$, $p < 0.001$; Fig. 2B) but was not related to the number of shoots (-0.08 ± 0.08, $z = -0.938$, $p = 0.348$).

Discussion

In this study, we evaluated trade-offs between sexual functions both at the individual flower level and at the whole plant level. Our results show that at the individual flower level, there is no relationship between the number of stamens and seeds produced. This suggests an absence of resource trade-offs between the two reproductive functions at the flower level. In contrast, at the whole plant level, there was a positive relationship between the number of stamens produced and the number of seeds. Taken together, these results indicate that there are no evident physiological trade-offs between female and male functions. The apparent lower probability of paternity with lower male sex expression was not compensated by a higher seed production in individuals that produced less than ten stamens per flower. In agreement, we did not find female function compensation of reduced male function in sexually intermediate *G. sylvaticum* plants in our previous work (Varga and Kytöviita 2016). These results suggest that the sexually intermediate phenotype may not be an evolutionary stable strategy unless other factors account for the apparent loss of male function.

To our knowledge, very few studies have investigated sex allocation trade-offs between pollen and seeds at the flower level (Devlin 1989; Mossop et al. 1994; Sato and Yahara 1999) and this is especially true for gynodioecious species (but see Atlan et al. 1992). All flowers in our study expressed the same potential for the female sexual function (i.e. contained a gynoecium with ten ovules) and only the male sexual expression varied. In the gynodioecious *Fragaria virginiana*, pollen production per flower has been shown to correlate negatively with the probability that a flower would develop into a fruit (Ashman 1999). In

9

contrast to our study species, hermaphroditic *F. virginiana* flowers do not present dichogamy. In the dichogamic protandrous *G. sylvaticum* flowers, the number of stamens is already developmentally fixed and irreversible at the time of ovule fertilisation, which could explain the lack of any relationship between stamen and seed number at the flower level. This further implies that the resource allocation to male function did not affect subsequent resource allocation to female function.

The above results were further corroborated at the whole plant level. The total number of stamens and seeds correlated positively at the whole plant level suggesting a lack of trade-off between the two sexual functions. Furthermore, the results indicate that resources are allocated in a manner where increasing flower number benefited both sexual functions similarly. Sex allocation between the female and the male functions has been extensively studied, especially in the context of size-dependent allocation, including gynodioecious plants (e.g. Ashman et al. 2001). Models assuming resource-limited female function predict a change towards higher seed production as plant size increases. There is experimental evidence for this and in many species female resource allocation is more responsive to plant size than male resource allocation (Dudash 1991; Koelewijn and Hunscheid 2000; Klinkhamer et al. 1994). In the present study, seed production was not correlated with the number of shoots produced, a proxy used for plant size. However, the male function (i.e. the number of functional stamens) increased with the number of shoots and, critically, the increase was similar in both functions (ß = 0.40± 0.06 vs. ß = 0.35 ± 0.09 for the male and female function, respectively). Therefore, we conclude that the plant size did not affect relative sex allocation in *G. sylvaticum.*

In theory, in species with cyto-nuclear gynodioecy, an increased allocation into male reproduction should be selected for in hermaphrodites (Maurice et al. 1993; Maurice et al. 1994; Schultz 1994). In the present study, the number of seeds per plant was higher in plants

that were nearly complete hermaphrodites (Fig. 3). It has been shown previously that seed production in *G. sylvaticum* is limited both by nutrient (Asikainen and Mutikainen 2005b) and light availability (Varga et al. 2015) in the field. However, it remains to be tested whether resource limitation is also responsible for stamen and ultimately, pollen production. We do know that light availability affects sex expression (i.e. the number of stamens produced) in this species even though we did not detect any significant effect on functional gender i.e. whether plants reproduced mainly through seeds or pollen (Varga and Kytöviita 2016).

To conclude, this study demonstrates the lack of marked trade-offs between the female and the male functions at the flower level and at the plant level in *G. sylvaticum*. The lack of measurable trade-offs suggests that there are no significant net costs of male restoration in this gynodioecious species. It is possible that hermaphrodites with reduced male function reproduce more through selfing to compensate for the loss of fitness as compared to outcrossing pollen donors. Selfing requires much smaller allocation to male function than out crossing (e.g. Whitton et al. 2008; Johnson et al. 2010). *Geranium sylvaticum* plants are self-compatible and inbreeding depression seems minor (Varga et al. 2013b) suggesting that the potential benefits of selfing could be high in this gynodioecious plant species.

References

Ashman T-L (1999) Determinants of sex allocation in a gynodioecious wild strawberry: implications for the evolution of dioecy and sexual dimorphism. J Evol Biol 12:648–661.

Ashman T-L, Majetic CJ (2006) Genetic constraints on floral evolution: a review and evaluation of patterns. Heredity 96:343–352.

Ashman T-L, Pacyna J, Diefenderfer C, Leftwich T (2001) Size‐ dependent sex allocation in a gynodioecious wild strawberry: the effects of sex morph and inflorescence architecture. Int J Plant Sci 162:327–334.

Asikainen E (2004) Maintenance of gynodioecy in *Geranium sylvaticum*. Ph.D. Thesis, Turku University.

Asikainen E, Mutikainen P (2003) Female frequency and relative fitness of females and hermaphrodites in gynodioecious *Geranium sylvaticum* (Geraniaceae). Am J Bot 90:226–234.

Asikainen E, Mutikainen P (2005a) Preferences of pollinators and herbivores in gynodioecious *Geranium sylvaticum*. Ann Bot 95:879–886.

Asikainen E, Mutikainen P (2005b) Pollen and resource limitation in a gynodioecious species. Am J Bot 92:487–494.

Atlan A, Gouyon P-H, Fournial T, et al (1992) Sex allocation in an hermaphroditic plant: the case of gynodioecy in *Thymus vulgaris* L. J Evol Biol 5:189–203.

Bailey MF (2002) A cost of restoration of male fertility in a gynodioecious species, *Lobelia siphilitica*. Evolution 56:2178–2186.

Bazzaz F and Grace J (1997) Plant resource allocation. Academic Press.

Caruso CM, Case AL, Bailey MF (2012) The evolutionary ecology of cytonuclear interactions in angiosperms. Trends Plant Sci 17:638–643.

Cody ML (1966) A general theory on clutch size. Evolution 20: 174–184.

Couvet D, Ronce O, Gliddon C (1998) The maintenance of nucleocytoplasmic polymorphism in a metapopulation: the case of gynodioecy. Am Nat 152:59–70.

De Haan AA, Hundscheid M, Van Hinsberg A (1997) Effects of CMS types and restorer alleles on plant performance in *Plantago lanceolata* L.: an indication for cost of restoration. J Evol Biol 10:803–820.

del Castillo RF, Trujillo S (2009) Evidence of restoration cost in the annual gynodioecious *Phacelia dubia*. J Evol Biol 22:306–313.

Devlin B (1989) Components of seed and pollen yield of *Lobelia cardinalis*: variation and correlations. Am J Bot 76:204.

Dudash MR (1991) Plant size effects on female and male function in hermaphroditic *Sabatia angularis* (Gentianaceae). Ecology 72:1004–1012.

Dufaÿ M, Vaudey V, de Cauwer I, et al (2008) Variation in pollen production and pollen viability in natural populations of gynodioecious *Beta vulgaris* ssp. *maritima*: evidence for a cost of restoration of male function? J Evol Biol 21:202–212.

Elzinga JA, Varga S (2017) Prolonged stigma and flower lifespan in females of the gynodioecious plant *Geranium sylvaticum*. Flora 226:72–81.

Johnson MTJ, deWitt Smith S, Rausher MD (2010) Effects of plant sex on range distributions and allocation to reproduction. New Phytol 186:769–779.

Klinkhamer P, de Jong TJ, Nell HW (1994) Limiting factors for seed production and phenotypic gender in the gynodioecious species *Echium vulgare* (Boraginaceae). Oikos 71:469.

Koelewijn HP (2003) Variation in restorer genes and primary sexual investment in gynodioecious *Plantago coronopus*: the trade-off between male and female function. Proc Royal Soc B 270:1939–1945.

Koelewijn HP, Hunscheid M (2000) Intraspecific variation in sex allocation in hermaphroditic *Plantago coronopus* (L.). J Evol Biol 13:302–315.

Levins R (1968) Evolution in changing environments. Princeton University Press, Princeton.

Lloyd DG (1980) Sexual strategies in plants III. A quantitative method for describing the gender of plants. New Zeal J Bot 18:103–108.

Maurice S, Belhassen E, Couvet D, Gouyon P-H (1994) Evolution of dioecy: can nuclear-cytoplasmic interactions select for maleness? Heredity 73:346–354.

Maurice S, Charlesworth D, Desfeux C, et al (1993) The evolution of gender in hermaphrodites of gynodioecious populations with nucleo-cytoplasmic male-sterility. Proc Royal Soc B 251:253–261.

Méndez M, Traveset A (2003) Sexual allocation in single-flowered hermaphroditic individuals in relation to plant and flower size. Oecologia 137:69–75.

Mossop R, Macnair MR, Robertson AW (1994) Within-population variation in sexual resource allocation in *Mimulus guttatus*. Func Ecol 8:410.

Obeso J-R (2002) The costs of reproduction in plants. New Phytol 155:321–348.

Parachnowitsch AL, Elle E (2004) Variation in sex allocation and male–female trade-offs in six populations of *Collinsia parviflora* (Scrophulariaceae sl). Am J Bot 91:1200–1207.

Renner SS (2014) The relative and absolute frequencies of angiosperm sexual systems: Dioecy, monoecy, gynodioecy, and an updated online database. Am J Bot 101:1588–1596.

Reznick D (1985) Costs of reproduction: an evaluation of the empirical evidence. Oikos 44:257–267.

Rivkin LR, Case AL, Caruso CM (2016) Why is gynodioecy a rare but widely distributed sexual system? Lessons from the Lamiaceae. New Phytol 211:688-696.

Ross M (1990) Sexual asymmetry in hermaphroditic plants. Trends Ecol Evol 5:43–47.

Sato H, Yahara T (1999) Trade-offs between flower number and investment to a flower in selfing and outcrossing varieties of *Impatiens hypophylla* (Balsaminaceae). Am J Bot 86:1699–1707.

Schnable PS, Wise RP (1998) The molecular basis of cytoplasmic male sterility and fertility restoration. Trends Plant Sci 3:175–180.

Schultz ST (1994) Nucleo-cytoplasmic male sterility and alternative routes to dioecy. Evolution 48:1933–1945.

Stearns SC (1989) Trade-offs in life-history evolution. Func Ecol 3:259–268.

Vaarama A, Jääskeläinen O (1967) Studies on gynodioecism in the Finnish populations of *Geranium silvaticum* L. Ann Aca Sci Fenn B 108:1–39.

Varga S (2014) Pre-dispersal seed predation in gynodioecious *Geranium sylvaticum* is not affected by plant gender or flowering phenology. Arthropod Plant Interac 8:253–260.

Varga S, Kytöviita (2010) Gender dimorphism and mycorrhizal symbiosis affect floral visitors and reproductive output in *Geranium sylvaticum*. Func Ecol 24:750–758.

Varga S, Kytöviita M-M (2016) Light availability affects sex lability in a gynodioecious plant. Am J Bot 103:1928–1936.

Varga S, Laaksonen E, Siikamäki P, Kytöviita M-M (2015) Absence of sex differential plasticity to light availability during seed maturation in *Geranium sylvaticum*. PLoS ONE 10:e0118981.

Varga S, Nuortila C, Kytöviita M-M (2013a) Nectar sugar production across floral phases in the gynodioecious protandrous plant *Geranium sylvaticum*. PLoS ONE 8:e62575.

Varga S, Vega-Frutis R, Kytöviita M-M (2013b) Transgenerational effects of plant sex and arbuscular mycorrhizal symbiosis. New Phytol 199:812–821.

Volkova PA, Rudakova VS, Shipunov AB (2007) Sex ratios in populations of *Geranium sylvaticum* in European Russia. Plant Spec Biol 22:125–128.

Whitton J, Sears CJ, Baack EJ, Otto SP (2008) The dynamic nature of apomixis in the angiosperms. Int J Plant Sci 169:169–182.

Figure captions

Fig. 1. Frequency distribution of the sexual expression in *Geranium sylvaticum* experimental

plants. The vertical line shows the mean. N = 196 plants.
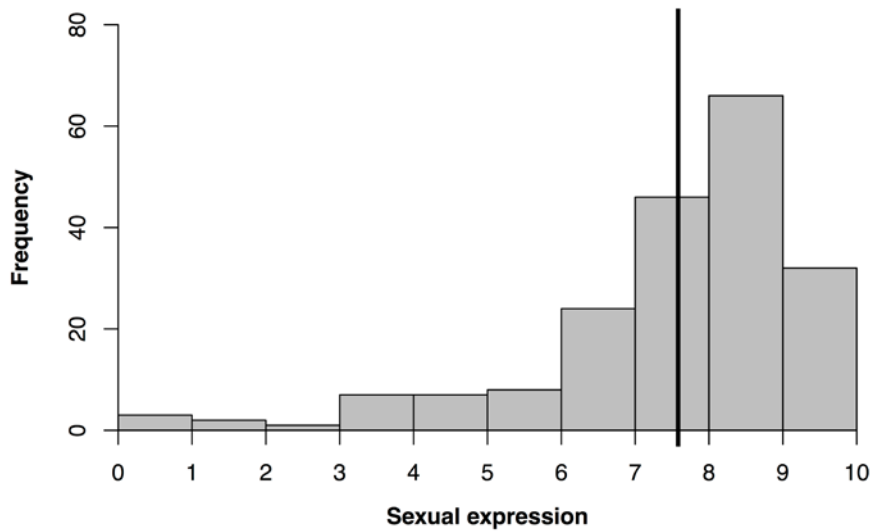

Fig. 2. Density distribution of the number of seeds vs. stamens produced at the individual (A)

flower and (B) plant level in experimental *Geranium sylvaticum* plants. N = 953 and N = 196
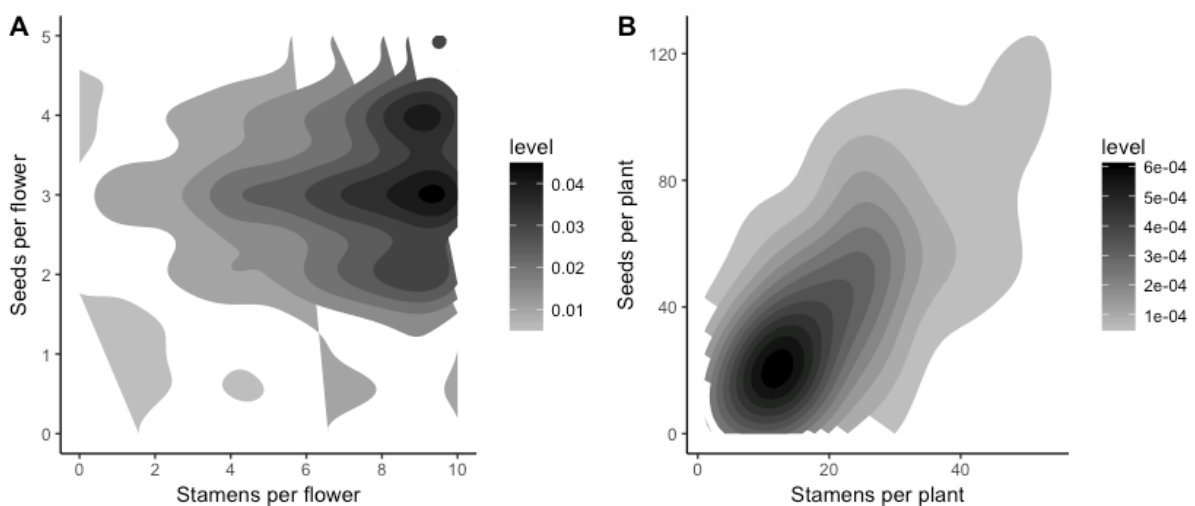
for flowers and plant respectively.

Fig. 3. Number of seeds produced in experimental *Geranium sylvaticum* plants in relation to plant sexual expression. Overlapping data points are plotted with random jitter for visibility. The line indicates the loess smoothed fit curves with a 95% confidence regions.