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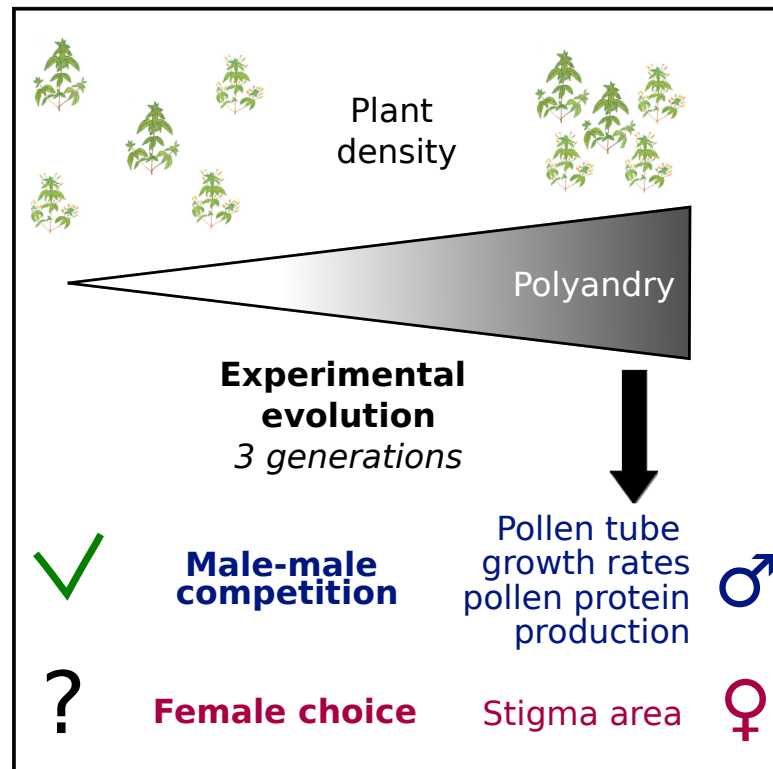
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Current Biology

Rapid evolution of pollen and pistil traits as a response to sexual selection in the post-pollination phase of mating

Graphical abstract



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In brief

Tonnabel et al. confirm the scope for sexual selection in the post-pollination phase of plant reproduction. Plants that evolved at a high plant density, which strengthens polyandry compared with low density, evolved faster-growing pollen tubes, upregulation of pollen proteins, and larger stigmas, which may conform to female choice processes.

Highlights

- High density promoted greater levels of polyandry among male and female plants
- Female plants at high density evolved enlarged stigmas
- Male plants at high density evolved faster-growing pollen tubes
- Several pollen proteins were upregulated at high density compared with low density

Report

Rapid evolution of pollen and pistil traits as a response to sexual selection in the post-pollination phase of mating

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SUMMARY

Sexual selection is the basis of some of the most striking phenotypic variation in nature.^{1,2} In animals, sexual selection in males can act on traits that improve access to mates prior to copulation,^{3–8} but also on sperm traits filtered by sperm competition,^{9–14} or female choice expressed simply by the morphology and physiology of genital tracts.^{14–16} Although long overlooked as a mode of selection on plant traits, sexual selection should act on land plants too because they are anisogamous: males produce more, and smaller, gametes than females.^{17–19} Numerical asymmetry in gamete production is thought to play a central role in selection on traits that affect pollen transfer to mates,^{20,21} but very little is known about how pollen competition or cryptic female choice might affect the evolution of traits expressed after pollination.^{22,23} Here, we report the divergence of pollen and pistil traits of the dioecious wind-pollinated annual herb *Mercurialis annua* during evolution over three generations between populations at low versus high plant density, corresponding to low versus higher levels of polyandry;²⁴ we expected selection under higher polyandry to strengthen competition among pollen donors for fertilizing ovules. We found that populations at high density evolved faster-growing pollen tubes (an equivalent of greater sperm velocity), greater expression of pollen proteins involved in pollen growth, and larger stigmas (a trait likely enhancing the number of pollen donors and thus competition for ovules). Our results identify the post-pollination phase of plant mating as an important arena for the action of sexual selection.

RESULTS AND DISCUSSION

A basic tenet of sexual-selection theory is that costly and complex male traits evolve in response to competition for access to sexual partners and their ovules.^{25–29} Cost and complexity imply that relaxing this competition should lead to a reduction in such traits, while intensifying it should lead to their augmentation. It has been well known since Darwin's³⁰ pioneering work that precopulatory sexual selection in animals underlies the evolution of male traits that promote their access to females via competition with other males, or of ornaments to seduce females into choosing them over other potential mates. The application of the same principle to post-copulatory selection predicts that sperm morphology, sperm motility, or seminal fluid content may respond to changes in the intensity of sperm competition,^{27,28} which has been empirically validated for several animals.^{4,9–16} In principle, sexual selection should act in any population displaying anisogamy, including plants, whose numerous

pollen grains often compete to fertilize far fewer ovules.^{17–19} It has long been recognized that sexual selection in plants can act on traits that increase the number of an individual's female-acting mates,^{17–21} but it might also act on pollen traits enabling an efficient fertilization of ovules through the growth of pollen tubes within pistils.^{22,23,31–33} Despite recognition of these general principles, the study of sexual selection in plants has remained disconnected from, and has lagged behind, theoretical and empirical studies in animals, with surprisingly little attention given to the evolutionary implications of variation in the number of sexual partners, a key metric in sexual-selection thinking.^{17–19,21}

To test for the effects of sexual selection during the post-pollination phase of plant mating, we took advantage of populations of the wind-pollinated plant *M. annua* that had diverged under experimental evolution in response to variation in the strength of sexual selection under low versus high plant density (see [STAR Methods](#) and [Figure S1](#) for a description of the methods).

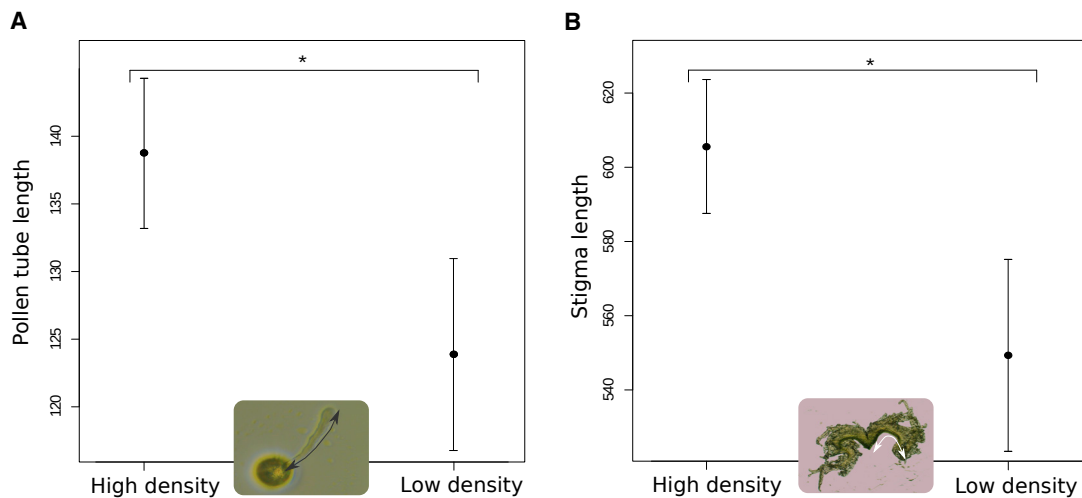


Figure 1. Pollen and pistil trait evolutionary responses to low versus high plant density after three generations of selection

Differences in pollen-tube length (A) and in stigma length (B) in experimental populations of *M. annua* that evolved over three generations at low versus high density, as inferred by our statistical models. Pollen-tube length, reflecting both pollen germination and pollen-tube growth rates, and stigma length were measured in a common garden and thus reflect evolutionary responses to plant density variation. Pollen-tube length and stigma length were treated as response variables; for pollen-tube length, the pollen density on the Petri dish was included as a fixed effect (see the main text for the structure of random effects). Pollen-tube and pistil lengths are unitless relative measures. The reported means correspond to the calculated means per treatment category, and the standard errors (horizontal bars) were estimated using our statistical models that account for experimental population as a random effect. The significance of differences between evolutionary treatments was evaluated using likelihood ratio tests (LRTs), comparing models with or without the treatment effect ($p < 0.05$, $df = 1$). See also Figure S1 and Table S3.

Specifically, populations evolving at contrasting densities diverged in terms of several measures of sexual dimorphism as a result of sexual selection during the pollination phase.^{21,24,34} Here, we turned our focus to the post-pollination phase of mating and asked whether the greater competition among pollen donors for access to ovules in high-density populations had favored the evolution of pollen traits that might enhance ovule fertilization compared with those in low-density populations. We thus measured pollen and pistil traits for the progeny of plants from our experiment after three generations of divergent selection.

Consistent with predictions from models of male-male competition,^{25–28} we found that males sampled from the high-density populations produced pollen with faster *in vitro* pollen-tube growth than those sampled from the low-density populations (with pollen-tube growth measured in terms of the speed of both pollen germination and pollen-tube growth over a period of 4 h on growth medium; Figure 1A; Table 1). Pollen-tube growth rate is generally considered to be a pollen competitive trait, and it has proven to be a good proxy for post-pollination fertilization success in controlled crosses in many plant species.^{31–33} The evolutionary divergence in pollen-tube growth rate between density treatments could be an artifactual effect on differences in pollen density, which is known to influence pollen-tube growth *in vitro*:^{34–36} if pollen production (and consequently the quantity of pollen transferred to the medium) differed among experimental populations, it may have altered pollen-tube development as a side effect. However, we did not observe any difference in pollen production between the density treatments, whether in terms of the mass of pollen harvested on eight plants or the number of flowers produced.³⁴ Moreover, whereas pollen density did affect the pollen germination rate *in vitro* in our study (e.g., Snow et al.³¹ and Skogsmyr and Lankinen³³), it did not

affect pollen-tube growth rates (Table 1). The divergence in pollen-tube growth rates between the two density treatments may thus be attributed to differences in the strength of post-pollination sexual selection experienced by the immediate ancestors of the plants we measured. If this inference is correct, our results complement and are consistent with the observations made on the insect-pollinated species *Collinsia heterophylla* in which one line maintained under polygamy evolved enhanced fertilization ability of pollen compared with a contrasting line that evolved under enforced monogamy.²²

In addition to divergence in the pollen-tube growth dynamics, we also observed divergence in the expression of several key proteins in the pollen produced by plants sampled from the two contrasting density treatments. We scanned the proteomes of pollen-coat-enriched protein extracts for systematic differences between experimental populations evolved at high versus low density, with all plants raised in a common garden after the three generations of divergent selection (STAR Methods). Of the 144 proteins from 674 peptides observed from mass spectrometry fragmentation data and with confidently annotated peptide sequences, we found ten that showed differential expression between the two density treatments. All ten were upregulated in the high-density populations ($p < 0.0019$), with substantial fold changes ranging from 1.7 to 17.4 (Tables 2 and S1; Figures 2A–2C). One of these upregulated proteins belonged to the twenty most abundant proteins, representing roughly 50% of the overall protein profile, while the other nine upregulated proteins showed a smaller relative abundance (Table S1; Figure S2). Four of the ten upregulated proteins are known to promote pollen development in *Arabidopsis thaliana*; four others are involved in pollen germination and/or pollen-tube growth; one protein is involved in both functions; and one is known to contribute to

Table 1. Evolutionary divergence in pollen and pistil traits evolving at low versus high plant density over three generations of selection and effect of pollen grain density on measured pollen traits

Plant trait	Intercept	Evolutionary treatment effect			Pollen density effect		
		B	χ^2	p	B	χ^2	p
♂ pollen germination probability	−0.611 (±0.518)	0.244 (±0.248)	0.847	0.36	−0.0159* (±0.00514)	9.47*	0.0021*
pollen-grain diameter	127 (±1.34)	−2.59 (±1.44)	3.28	0.070	−0.0659 (±0.0647)	1.06	0.30
pollen-tube length	138 (±5.54)	−14.9* (±7.09)	4.19*	0.041*	−0.147 (±0.177)	0.720	0.40
♀ stigma length	604 (±17.8)	−55.4* (±25.4)	4.65*	0.031*	–	–	–
style length	121 (±7.10)	−12.1 (±10.1)	1.65	0.20	–	–	–
maximum width of stigma	95.5 (±2.86)	−7.00 (±4.11)	2.91	0.088	–	–	–
stigma hair length	308 (±13.7)	−0.569 (±19.6)	0.00240	0.96	–	–	–

Pollen and pistil traits were assessed in a common garden, at a fixed plant density, in ten experimental populations that had previously evolved independently at low or high density (five populations each). Pollen traits were measured using an *in vitro* growth medium in different microscope slides, thus allowing us to assess the effect of pollen density on the slide on pollen traits. The effect of both the evolutionary treatment and pollen density was tested on the basis of model comparisons using LRTs to compare models with and without the effect. The null models predicted each response variable as a function of the following fixed effects: the evolutionary treatment (with high density as reference); and, for pollen traits only, pollen density (see the STAR Methods section for a description of the structure of random effects). Beta refers to the estimate of the difference between evolutionary treatments (low density – high density) or of the linear slope on pollen density. The degrees of freedom were equal to one in all model comparisons. Asterisk (*) represents significant p values. See also Figure S1 and Table S3.

plant responses to water stress, and to be expressed in pollen³⁷—though with a yet unidentified specific role (Tables 2 and S1 and references therein). The most abundant proteins in *M. annua* pollen are known to play a role in pollen development and pollen-tube growth in *A. thaliana* (Table S2). In addition to identifying these ten proteins, the overall protein expression diverged qualitatively between density treatments in our experiment: Figure 2D illustrates a separation between our density treatments along the first ordination plan, explaining 61.9% of variation in peptide abundance. Collectively, these results demonstrate not only the rapid evolution of the expression of proteins known to be involved in pollen competitiveness but also that plant density has implications for the overall expression of proteins in the pollen coat, with a potential effect on the level of polyandry.

Sexual selection may also affect the evolution of female traits relevant to “choosiness.” For instance, under Fisher’s “runaway” process, female choosiness may coevolve with male display or male ornaments as a genetically correlated response,^{40,41} the female preference and preferred male trait being jointly transmitted to progeny. Female choosiness may also contribute toward ensuring that progeny inherit “good genes.”^{41,42} While these predictions have been shown to apply to traits involved in precopulatory sexual selection,^{43,44} similar coevolutionary dynamics can also take place at the post-copulatory stage.^{45,46}

In many plant species, mate choice occurs through the filtering of pollen tubes bearing specific incompatibility alleles in self-incompatible plants,⁴⁷ but the possibility that pistil morphology or physiology also filters pollen tubes based on their genetic or fertilization qualities has been largely overlooked to date (but see Sarkissian and Harder⁴⁸ and Marshal and Ellstrand⁴⁹). This is surprising, as it is well established for animals that genital traits can conform to female choice models by biasing paternity for specific sperm traits,^{9–14,50,51} and the extent to which pistil tissues might contribute to selection of particular pollen traits has been identified as a key question in the study of sexual selection

in plants.²³ Simple pistil traits may intensify the post-pollination component of sexual selection by increasing the number of pollen donors that effectively compete for ovules, as is the case for female genital traits in animals. Larger stigmas have been shown in other plant species to allow the accumulation of more pollen grains,^{52,53} which may contribute to enhancing the diversity in paternity. Longer styles may select pollen traits by increasing the distance to reach ovules. We thus asked whether differences in the degree of post-pollination sexual selection between the two different density treatments in our experiment could have promoted divergence in the morphology of pistils in females.

Our results revealed that stigmas of female progeny sampled from the high-density populations of our experiment were larger than those from the low-density populations (Figure 1B; Table 1). The evolution of larger stigmas in a wind-pollinated species might be expected under conditions of pollen limitation. However, we found no evidence of pollen limitation in any of our populations,⁵⁴ and the greater size of stigmas of females from the high-density treatment in any case runs counter to expectations for divergence due to differences in pollen limitation. Our observation of the evolution of larger stigmas at the high density compared with those at low density is thus consistent with a form of female choice acting during the post-pollination phase of plant mating.

Our previous work has shown that the pollen is effectively dispersed to pistils of females from a greater number of males at high density compared with that at low density.²¹ Given this opportunity to capture pollen from a greater diversity of males, females may benefit from evolving larger stigmatic surfaces by intensifying competition among pollen tubes to reach ovules, ultimately transmitting genes to progeny that, in turn, are better able to compete for fertilization success. Thus, a “runaway” process would involve passing genes to progeny that code both for larger stigmas and for higher pollen competitiveness. Additionally, pollen competitiveness may coincide with higher genetic quality for fitness traits in general, especially given that genes expressed in pollen tubes are largely also expressed in sporophytes.⁵⁵ Larger

Table 2. Upregulated pollen proteins in experimental populations that evolved at high density compared with low density

Accession ^a	<i>A. thaliana</i> ortholog ^b	Protein functional annotation	No. peptides ^c	Confidence score ^d	ANOVA (p value)	Fold change
g11745	At4g09740	endoglucanase-like	2	85	0.0024	3.96
g18719	At4g20050	polygalacturonase QRT3	2	98	0.0108	16.68
g14342	At2g22780	malate dehydrogenase, glyoxysomal	2	124	0.0216	8.68
g29109	At1g07380	neutral ceramidase 1-like	3	162	0.0219	4.12
g36421	At1g47980	desiccation-related protein PCC13-62-like	3	121	0.0233	1.68
g16088	At5g16920	FAS1-domain-containing protein SELMODRAFT_448915	5	407	0.0243	17.39
g15255	At4g12390	putative-pectinesterase-inhibitor-domain-containing protein	5	215	0.0246	2.95
g20031	At1g66970	glycerophosphodiester phosphodiesterase GDPDL3-like	2	97	0.0311	11.68
g13338	At3g07970	polygalacturonase QRT2-like	2	62	0.0356	2.56
g20613	At3g29075	glycine-rich protein	2	123	0.0491	6.17

Summary of the main characteristics of the ten pollen proteins identified as upregulated in the high- versus low-density treatment (full functional characterization is available in [Table S1](#)). p values were corrected for multiple testing using a false discovery rate correction accounting for the full list of identified proteins. See also [Figure S1](#) and [Table S1](#).

^aOrtholog identification name in the annotation of the *Mercurialis annua* genome³⁸

^bClosest ortholog in *Arabidopsis thaliana* obtained with TAIR BLAST (V2.9.0+)³⁹

^cNumber of peptides used for protein quantification

^dScore resulting from Mascot protein identification search ([STAR Methods](#))

stigmas may therefore be selected because they allow females to pass on “good genes,” thereby ensuring a high genetic quality in their offspring, too. Testing the validity of “runaway” and “good genes” models during pollen-pistil interactions will require future evaluation of whether evolved pistil traits (here stigma size) bias paternity for evolved pollen traits (here faster pollen tubes), and whether genetic correlations have evolved between the two sets of traits.

Taken together, our results demonstrate that pollen and pistil traits can evolve rapidly under experimental evolution, just as traits that affect pre-pollination processes may do.^{56–60} Previous work on responses to sexual selection in *M. annua*, in particular, has identified rapid evolutionary responses to changes in plant density for various morphological traits that affect mate acquisition during the pre-pollination phase, though no change was observed in the amount of pollen produced.³⁴ Those vegetative traits are likely to have evolved in response to selection in the context of both altered mating conditions and altered conditions of competition for light. In contrast, it seems unlikely that the evolutionary response reported here, affecting post-pollination mating success, was significantly influenced by competition for resources at the plant level. Indeed, any such influence would have been opposed to that inferred for our experiment, i.e., with increased resource limitation at high density likely causing plants to reduce rather than increase allocation to costly reproductive traits.

The rapid evolution of both male and female post-pollination traits is reminiscent of similar findings in animals in which polyandry affects the evolution of post-copulatory male-female interactions (reviewed in Firman et al.¹⁴). However, reproduction in plants does not involve the one-to-one interactions that are typically the basis of pre-copulatory sexual selection in most

animals, including species in which females chose whether or not to copulate after direct interactions with their potential mates.¹⁴ In wind-pollinated plants such as *M. annua*, ecological factors such as plant density or wind direction presumably play an important role in determining the degree of polyandry,^{21,24} as would be the case for density and water flow in animals with external fertilization such as broadcast spawners.^{61–64} Our previous work on pre-pollination sexual selection in *M. annua* indeed suggests obvious parallels with its action in broadcast spawners, with male-male competition at different densities affecting the evolution of different plant vegetative traits involved in pollen dispersal.^{21,24,34} Unlike broadcast spawners, however, the post-pollination component of sexual selection of angiosperms, which does involve physical one-to-one interactions (between male gametophytes and female sporophyte), is likely to involve sexual selection with mechanisms similar to animals with internal fertilization.

In conclusion, by illustrating the potential for post-copulatory sexual selection in plants, our study broadens the empirical base for tests of sexual-selection theory. The evolutionary responses observed for both pollen and pistil traits highlight the likely role of multiple mating in plants in shaping plant traits through post-pollination sexual selection.⁶⁵ The rapid evolution of traits expressed at the post-pollination stage in both pollen and pistil tissues in response to density, a simple demographic variable that tends to vary substantially over space and time, suggests that such mechanisms are likely to be in a constant state of evolutionary fine-tuning in nature. Empirical evidence has been accumulating from studies on animals that sexual selection contributes to purging mutation load and thus to improving population fitness through the selection of individuals with overall better condition.^{66–68} Our results imply that such a

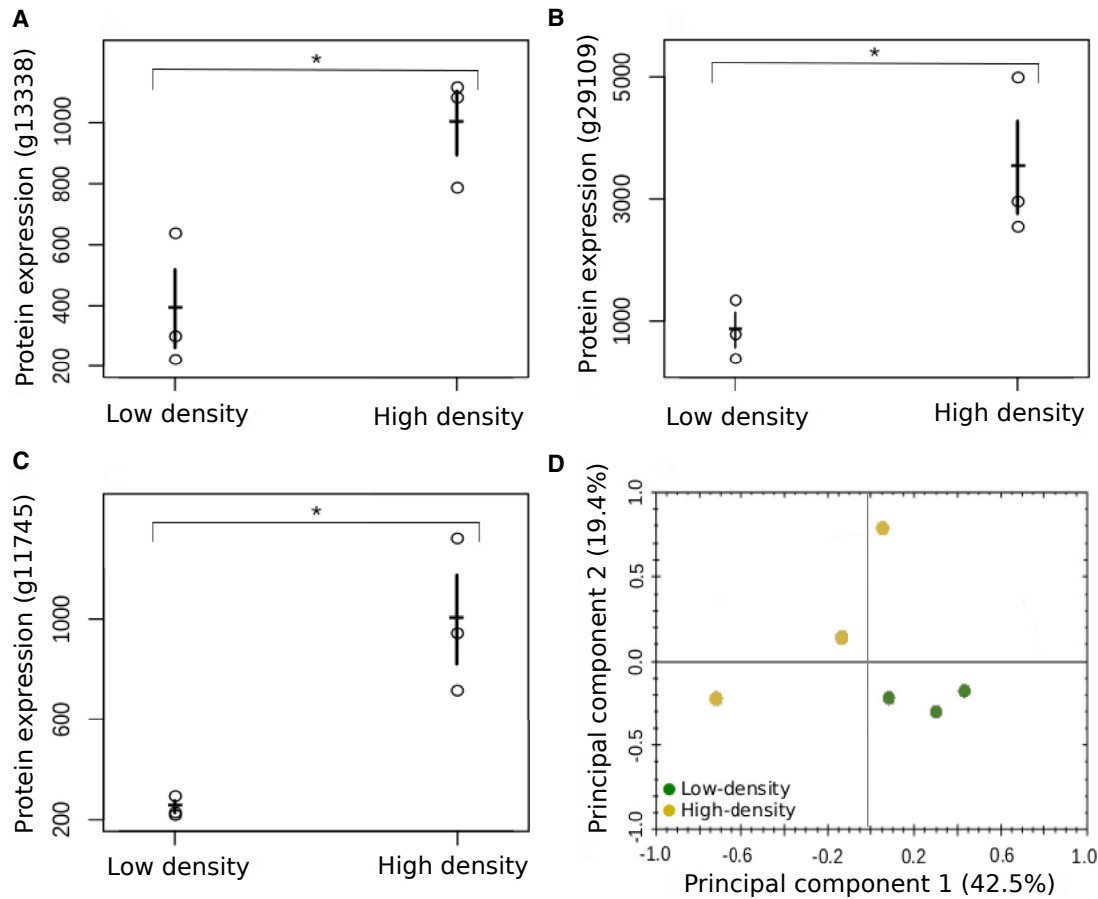


Figure 2. Divergence in pollen protein production between populations evolving at low versus high plant density over three generations of selection

Protein abundance differences between experimental populations of *M. annua* that evolved over three generations at low or high density for three of the ten proteins that were found to be upregulated at high density (A, g13338 accession in the *M. annua* genome; B, g29109; C, g11745; * $p < 0.05$). The average protein abundance is shown by the horizontal bar, with the vertical bar expressing one standard deviation within each density treatment; protein abundance pooled across 28 males per experimental population are displayed by empty circles. The overall difference between density treatments (D, low density in green and high density in yellow) is described by a principal-component analysis performed using raw data on peptide abundances. The first ordination plan, explaining 61.9% of variance in protein expression, allows differentiation between density treatments. See also [Figure S2](#) and [Tables S1](#) and [S2](#).

process of genetic purging may apply to plant populations not only from selection during the pollination phase of mating but also after pollen has been deposited successfully on receptive stigmas.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- Label-free protein quantification
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- Statistical analysis of the pollen and pistil phenotypes

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cub.2022.07.077>.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.T., P.D., and J.R.P.; methodology, J.T., P.C., A.L., J.-C.M., P.D., and J.R.P.; formal analysis, J.T., P.C., A.L., J.-C.M., and M.A.B.M.; investigation, J.T., M.A.B.M., and L.G.; writing – original draft, J.T.; writing – review & editing, P.C., A.L., J.-C.M., P.D., and J.R.P.; supervision, P.D. and J.R.P.; funding acquisition, J.T. and J.R.P.

DECLARATION OF INTERESTS

L.G. currently works at the University of Zagreb.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Experimental evolution lines of <i>M. annua</i> evolved at high or low density for three generations	Tonnabel et al. ³⁴	N/A
Chemicals, peptides, and recombinant proteins		
Agarose (1.5%)	Sigma	N/A
CaCl ₂	N/A	N/A
MgSO ₄	N/A	N/A
KCl	N/A	N/A
H ₃ BO ₃	N/A	N/A
Sucrose	N/A	N/A
Tris-HCl, pH 8.8	N/A	N/A
Sodium dodecyl sulphate (SDS)	N/A	N/A
Dithiothreitol (DTT)	N/A	N/A
Buffered phenol, pH 8.0	Sigma	N/A
Trichloroacetic acid	N/A	N/A
Cold acetone	N/A	N/A
Urea	N/A	N/A
Thiourea	N/A	N/A
CHAPS	N/A	N/A
C7BzO	N/A	N/A
Tributylphosphin	N/A	N/A
Polyacrylamide gel	N/A	N/A
Coomassie blue	N/A	N/A
Iodoacetamide	N/A	N/A
Trypsin	Promega	N/A
TFA	N/A	N/A
Acetonitrile	N/A	N/A
Formic acid	N/A	N/A
Deposited data		
<i>M. annua</i> genome	Veltsos et al. ³⁸	https://www.ebi.ac.uk/ena/browser/view/GCA_937616625
TAIR proteomic database for <i>Arabidopsis thaliana</i>	Garcia-Hernandez et al. ³⁹	https://www.arabidopsis.org/
Software and algorithms		
ImageJ software	Schneider et al. ⁶⁹	https://imagej.nih.gov/ij/
Progenesis QI software, version 2.2.04	Waters	https://www.waters.com/waters/fr_FR/Progenesis-QI-Software/
R software version 3.6.3	R Core Team ⁷⁰	https://www.r-project.org/
lme4 R package	Bates et al. ⁷¹	https://cran.r-project.org/web/packages/lme4/index.html

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mascot algorithm, Matrix Science, version 2.2.04	Perkins et al. ⁷²	N/A
Other		
Microscope EVOS XL Core	N/A	N/A
Hybrid linear ion trap-orbitrap mass spectrometer LTQ-Orbitrap Elite	Thermo Scientific	N/A
Liquid nano-chromatography system, Easy-nLC II	Thermo Scientific	N/A
Enrichment column, C18 PepMap100	Thermo Scientific	N/A
Reversed-phase column, NTCC-360/100-5-153	NikkyoTechnos	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests about the dataset or methods should be sent to, and will be dealt with by, the lead author, Jeanne Tonnabel (jeanne.tonnabel@cnrs.fr).

Material availability

This study did not generate novel biological material.

Data and code availability

The datasets corresponding to the evolutionary trajectories of both pistil and pollen traits have been deposited to the Mendeley repository (<https://doi.org/10.17632/v9rsxb8yt2.1>) and are publicly available as of the date of publication. The output of the protein quantification is available upon request from the lead author. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mercurialis annua is an annual wind-pollinated herb with a wide distribution in Europe and around the Mediterranean Basin.⁷³ The species complex includes dioecious, androdioecious and monoecious populations in different parts of its range.^{74,75} To establish our experimental evolution populations, we used dioecious plants of *M. annua*, which display sexual dimorphism in both morphology^{54,76} and gene expression.⁷⁷ Both sexes start producing flowers shortly after seed germination, and reproduction continues indeterminately over a period of three to four months.⁷⁸

We pooled seeds from 35 populations of *M. annua* from a metapopulation in northeastern Spain, with approximately 30 females sampled per populations (see Tonnabel et al.³⁴ and Pannell⁷⁸), i.e., our seed sample represents the genetic and phenotypic variation among populations linked by migration and recolonization following local extinctions.^{79,80} Prior to setting up our experiment we allowed individuals to mate freely over three consecutive generations (from 2012 to 2014) in a common garden in Lausanne to eliminate maternal effects, enhance standing genetic variation and to reduce genetic correlations that may have emerged from trait combinations prevailing in different parts of the sampling area. Any putative genetic correlation between pollen, pistil and fitness traits expected by 'runaway' or 'good-genes' models would not have been affected by these generations of open-pollination, given that paternity biases within pistils were still possible. Hereafter, we refer to the seed pool retrieved after these three generations as the 'source population'.

METHOD DETAILS

Experimental evolution protocol

Our protocol followed classical procedures of experimental evolution in which natural selection acts on standing genetic variation in the source population⁸¹ but under contrasting plant density treatments. Using well-mixed seeds from the source population, we established ten experimental populations, five assigned randomly to a high-density and five to a low-density treatment (Figure S1; see Tonnabel et al.^{24,34,54} for details on the growing procedures). Experimental populations were separated by 20 meters to ensure that

reproduction occurred independently in each of them; our previous studies estimated the *M. annua* pollen dispersal kernel for the same experimental design, revealing that most pollen disperses within a few meters of the plants and that very few pollen grains immigrate successfully into populations from more than several meters away.¹⁶ Dense vegetation was allowed to grow in between the experimental populations as a further barrier to gene flow among them. Individuals in each experimental population were allowed to mate freely with one another over three generations, with consecutive generations established from seeds from the parents of the corresponding replicate (i.e., each experimental population evolved independently). Pollen limitation was not expected at either density, based on knowledge accumulated on the pollination biology of *M. annua*.^{24,82}

Each generation, we first grew plants indoors in separate pots until they could be sexed. We then formed male-female pairs by transplanting a male and a female plant into each pot, which were placed outside. Each experimental population comprised 100 such male-female pairs. Pots in all populations were first established at low density (1 m between pots in a square grid) until plants had reached full maturity. At that point, all pots in all replicates were moved to a new randomly chosen position in the population. For populations of the low-density treatment, the initial low-density spacing was maintained, whereas pots in the high-density treatment populations were moved closer together, with a reduced between-pot distance of 0.2 m (measured from rim to rim). Plants were then allowed to release any seeds that had already been sired, and to continue mating. After four more weeks (by which time all seeds in fruits on the plant would have been sired under the experimental conditions), plants were harvested, threshed, and all seeds were mixed within each experimental population; the next generation was sown from a random sample of this seed mix. The mixing ensured that the expected contribution of each plant to the following generation was proportional to the number of seeds it produced or sired.

Establishment of a final common garden

After populations had evolved independently over three generations, we established progeny from the third generation in a common garden in greenhouses at the University of Lausanne, Switzerland (Figure S1). Seeds from each of the experimental populations were germinated following the same growing protocol as described above for the experimental evolution.³⁴ Month-old mature plants were sexed and 20 females and 30 males for each of the ten experimental populations were transplanted in separate pots (1 L, 14 cm diameter, 10.6 cm depth) using commercial soil (Ricoter 140). The initial difference between the sexes in the number of plants grown simply reflected the need to measure both macroscopic traits and protein expression in pollen in males compared to only pistil traits in females. Due to space constraints within the greenhouses, re-potted plants were grown at a high density in the common garden. After their transplantation, males and females were separated in two greenhouses to prevent any pollen dispersal to pistils that would elicit changes in pistil morphology. In each greenhouse, plants were randomly positioned and allowed to grow for an additional four weeks. At this stage, we harvested pistils and pollen from individual plants to assess their morphology and aspects of pollen-tube growth (see below). We randomized experimental populations when sampling pollen and pistils, and when measuring them (see below). Four persons were involved in the harvest while two persons respectively measured pollen and pistils to minimize any observational bias.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantifying the evolution of pollen morphology and pollen-tube growth rates

To investigate the evolution of pollen-tube growth from plants evolved at contrasting densities, we harvested pollen from dehiscent anthers of several males from the replicate experimental populations and observed pollen tubes growing on a nutritive agar medium. To prepare the growth medium we dissolved agarose (Sigma, 1.5%) with 5 mM CaCl₂, 1 mM MgSO₄, 5 mM KCl, 0.05% H₃BO₃, and 10% sucrose. The optimal growth medium for *M. annua* pollen tubes had been assessed previously by varying the H₃BO₃ and sucrose concentrations and examining pollen-tube growth. We chose the combination of boric acid, sucrose concentration and growing duration (i.e., four hours) that best allowed us to discriminate pollen-tube growth dynamics among males. A 50 μL drop of the agarose growing media was deposited on a microscope slide. The microscope slide was placed in a Petri dish containing a wet filter paper (Whatman) to maintain humidity favorable to pollen germination. For each male, pollen grains were harvested from the first six male inflorescences on the main plant axis that contained dehiscent anthers. For each inflorescence, pollen was powdered onto a separate microscope slide. Following pollen harvest, Petri dishes were incubated in a phytotron (Percival) at 22°C in the dark. After four hours incubation, we took an image of each slide using a camera coupled to a microscope (EVOS XL Core), using the same 4x magnification for all images. The length of pollen tubes measured after four hours of incubation therefore reflects differences in the speed of both pollen germination and pollen-tube growth.

We used ImageJ⁷⁹ to measure: (a) the number of pollen grains that had germinated; (b) the number of pollen grains that did not germinate; (c) the size of five randomly chosen pollen grains that had not germinated; and, (d) the length of five randomly chosen pollen tubes (Figures 1 and S1). We considered that a pollen grain had germinated when the pollen tube was longer than half of the pollen grain diameter. We also recorded the number of pollen grains for which germination status could not be assessed in order to calculate pollen-grain density on the slide; pollen-grain density is known to affect pollen germination rates⁸³ and needs to be taken into account as a co-factor in statistical analyses (see below). The viability of pollen grains that did not germinate was not assessed, so that the reported lack of treatment differences regarding pollen size (Table 1) may simply reflect similar sizes of non-viable pollen. Pollen measurements had to be spread over two weeks owing to space constraints for growing pollen tubes within the phytotron. We therefore recorded two temporal blocks corresponding to these two weeks. We discarded some pictures because we could not

properly measure pollen sizes, either because of the poor resolution of the picture or because a high density of pollen grains prevented assessing the number of germinated and non-germinated pollen grains. Our final pollen dataset for germination rate estimates contained between 4 and 12 males per experimental population (mean 8.5; 85 males in total), including between 24 and 72 inflorescences sampled per population (mean 51; 510 inflorescences in total; [Table S3](#)). The pollen-grain germination rate was computed on the basis of the observation of a total of 7,320 pollen grains, while traits were measured on 1,297 pollen grains and 2,083 pollen tubes. The resulting pollen and pistil morphological dataset is available on Mendeley (<https://doi.org/10.17632/v9rsxb8yt2.1>).

Pollen protein extraction for quantification

We further asked whether any observed changes in pollen macroscopic traits were accompanied by changes in pollen coat protein abundance. We adopted proteomic protocols to identify and quantify the protein expression in pollen coat-enriched protein extracts from experimental populations that had evolved at low versus high density. We first collected fresh pollen from 24 plants per experimental population, divided into three samples (each from eight males). On each plant, fresh pollen was collected from all dehiscent anthers, thus encompassing anthers of various ages including some anthers that had already dispersed fertile pollen. Each pollen sample originating from eight males was weighted prior to the pollen coat-enriched protein extraction. The resulting weight of pollen therefore stands as a proxy for pollen production at one point in time, and may be enriched in sterile pollen. We extracted proteins from these pollen samples with a protocol aimed at enriching extracts in pollen-coat proteins, while still retrieving proteins from the pollen grain (see detailed method below). We then pooled all three pollen coat-enriched protein extracts per experimental population to obtain a sufficient quantity of proteins for subsequent mass spectrometry. Six experimental populations (i.e., three from each plant density) yielded sufficient protein for further analysis. Statistical analyses of protein expression was thus carried out at the scale of experimental populations, with samples corresponding to an average expression of proteins in pollen coats sampled on 24 males. For each experimental population separately, we first migrated each protein sample on a polyacrylamide gel to concentrate the protein extracts into a single gel band, which was then cut and submitted to a trypsin digestion to cleave each protein extract into peptides. Each protein sample consisting of a single gel band before trypsin digestion thus corresponded to proteins produced by males in a single experimental population, and each gel band was kept separate for further analyses. Each resulting peptide mixture corresponding to a digested gel band was then analyzed using LC/MSMS mass spectrometry, allowing us to obtain the sequence of each peptide by fragmenting peptides into several smaller units and studying their mass relative to that of the known mass of amino acids.

Three weeks after the onset of pollen measurements, we collected pollen material from experimental populations that had evolved at low and high density. We harvested and weighed pollen from all dehiscent anthers found in flowers of 24 males in each experimental population, by groups of eight males. In each population, we pooled the fresh pollen collected on eight male plants and performed the protein extraction on these pooled samples (i.e., three samples per population). This harvest of fresh pollen material resulted in some differences between experimental populations in the pollen weight harvested overall ([Table S3](#)). Because potential differences in pollen production between experimental populations might elicit a treatment-specific bias in protein detection, we first checked that pollen production did not vary between treatments. We compared these weights of fresh pollen grains between our density treatments. To do so, we constructed a simple linear model explaining the total pollen weight collected on eight males between treatments, and compared this model with a model that excluded treatment as a fixed effect, using a LRT (using the R software, version 3.6.3⁷⁰ and the lme4 package⁷¹). We treated populations as a random effect in the compared models to account for the fact that populations, but not individuals within populations, consist of independent observations. We detected no difference in pollen weight between density treatments ($X^2 = 0.0041$, $df = 1$, $p = 0.98$).

Pollen-coat protein extractions ([Figure S1](#)) were carried out using a modified version of standard protocols.^{84,85} Pollen-coat protein extractions were performed separately for pollen harvested on groups of eight male plants corresponding to a pollen weight ranging from 6.8 mg to 37 mg (mean 20 mg; [Table S3](#)). Each sample of fresh pollen was placed on a Whatman N° 54 filter paper positioned on a mortar. In each mortar, we added 10 mL of chloroform in which we gently shook pollen grains for two minutes to minimize pollen burst during the protein extraction. This procedure allowed the extraction of proteins originating from the pollen coat, but a fraction of the proteins extracted would nonetheless result from the pollen grain itself. The pollen was separated from the solvent with the removal of the filter paper. The chloroform containing pollen-coat proteins and lipids was then evaporated under a fume hood. Proteins were extracted by homogenized the resulting material in the mortar using 1.0 mL 0.25 M Tris-HCl, pH 8.8, 2 % sodium dodecyl sulphate (SDS), and 20 mM dithiothreitol (DTT). After its transfer into 2.0 mL tubes, each sample was centrifuged at 15 000 g for 10 min. The supernatant was mixed with an equal volume of buffered phenol (Sigma, pH 8.0), which was vortexed for 2 min. The resulting homogenates were centrifuged at 15 000 g for 5 min. The phenolic phase, which contained proteins, was transferred to new 2.0 mL tubes. In each tube, 1 mL of acetone containing 10% w/v trichloroacetic acid and 65 mM DTT was added and incubated at -20°C overnight, allowing protein precipitation. After a centrifugation at 10 000 g for 45 min, the pellets were twice suspended in cold acetone by 10 000 g centrifugation for 10 min. After air-drying, pellets containing proteins were stored at -20°C.

Protein concentration and enzymatic digestion

For global proteolysis to generate the corresponding complex peptide mixtures ([Figure S1](#)), we dissolved protein extracts in 60 μ L of solubilisation buffer (urea 7 M, thiourea 2 M, CHAPS 2 %, C7BzO 0.5 %, DTT 20 mM, tributylphosphin 2 mM). At this stage, all three pollen coat-enriched protein samples belonging to the same experimental population (and corresponding to the fresh pollen of eight males) were pooled to maximize the quantity of protein, a necessary procedure for their subsequent identification. Each protein

sample (corresponding to a single experimental population) was loaded into a 7% polyacrylamide gel for a 90 min migration at 10 mA/V gel to concentrate the whole protein extract into a single gel band. The gel was stained with Coomassie blue to detect the single band containing all the proteins (corresponding to pollen harvested from 24 males in a single experimental population). At this stage, samples from two populations for each density treatment had < 5 μg of protein and had to be discarded from further analyses; the following analyses were therefore carried out on three replicate populations from each of the two density treatments and corresponded to the harvest of pollen material on a total of 144 plants. Each protein sample consisting in a single gel band thus corresponded to an experimental population and was kept separated for further analyses. After Coomassie blue staining, the revealed protein gel bands were excised and immersed first in a reductive buffer (5 mM DTT), and subsequently in an alkylating buffer (20 mM iodoacetamide). Following washing steps, gel bands (one per sample) were submitted to protein digestion using 1 μg of trypsin (Promega), which resulted in proteolytic cleavage into peptides. After overnight incubation at 37 °C, several steps of peptide recovery were performed using 0.1% TFA and acetonitrile. Finally, for each sample, these peptide fractions were combined and dried.

Mass spectrometry for peptide identification

We used mass spectrometry to identify peptide sequences produced by the previous proteolysis of the protein extracts (Figure S1). Peptide mixtures were dissolved in a 0.1% formic acid solution. All samples were analyzed with a hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap Elite, Thermo Scientific) equipped with a nano-ESI source and coupled to a liquid nano-chromatography system (Easy-nLC II, Thermo Scientific). Each sample was first loaded onto an enrichment column (C18 PepMap100, Thermo Scientific), and the separation was performed on a reversed-phase column (NTCC-360/100-5-153, NikkyoTechnos). The gradient (mobile phase A, H₂O/0.1% formic acid (FA); mobile phase B, CH₃CN/0.1% FA) was delivered at a flow rate of 300 nL/min. Tryptic peptides were eluted from the reversed-phase column into the mass spectrometer, using a linear gradient from 2 to 40% in 105 min, followed by a rapid increase to 80% in 4 min and a final 15 min isocratic period at 80%. The mass spectrometer was operated in the data-dependent mode with a typical 'Top 20' method. The first scan (MS spectra) was recorded in the Orbitrap analyzer (R = 60,000) with the mass range m/z 400-1,800. The 20 most intense ions were then selected for MS2 experiments, excluding singly charged species. Dynamic exclusion of already fragmented precursor ions was applied for 30 s, with a repeat count of two, a repeat duration of 30 s, and an exclusion mass width of \pm 5 ppm. The precursor isolation width was 2 m/z . Fragmentation occurred in the linear ion trap analyzer with normalized collision energy of 35. All measurements in the Orbitrap analyzer were performed with on-the-fly internal recalibration (lock mass) at m/z 445.12002 (polydimethylcyclsiloxane). This mass spectrometry methodology allowed us to obtain the sequence of each peptide (MS/MS spectra; Figure S1) by fragmenting peptides into several smaller units and studying their mass relative to that of the known mass of amino acids.

Label-free protein quantification

To quantify protein abundance, a label-free experiment was designed following previous methods.⁸⁶ Raw mass spectrometry data were imported into the Progenesis LC-MS software (V4.0.4441.29989, Nonlinear Dynamics). In this software, the most representative LC-MS run was set as a reference, and the retention times of all of our peptide extract separations were aligned for their later comparison. Raw peptide abundances were then normalized. We compared peptide-normalized abundance between low- and high-density populations, separately for each peptide using one-way analysis of variance (ANOVA) that included one value of expression per experimental population combining the expressed proteins of 24 male plants. We then restricted our analysis to peptides, hereafter referred to as 'selected peptides', for which we found significant abundance differences between the low- and high-density treatments. Using the Mascot algorithm (Matrix Science, version 2.2.04),⁷² peptide fragmentation data (MS/MS spectra) were compared to the list of proteins generated from the computed translation of the genome of *Mercurialis annua*.³⁸ This list of proteins contains the protein sequences in fasta format obtained from the translation of genome of *M. annua*, which are then subject to a simulated trypsin digestion. To match amino acid sequences of our peptide extract samples to that of the computed translation of the *M. annua* genome, we adopted the following criteria: (1) we allowed for one missed cleavage during trypsin digestion, and (2) we allowed for variable modifications that may result from carbamidomethylation of cysteine and oxidation of methionine. Mass tolerances for precursor and fragment ions were set at 5 ppm and 0.35 Da, respectively. MS/MS data resulted in table containing the set of peptides with an intensity value. These MS/MS data were first subjected to an identification step by the Mascot software (using a Decoy database, we set an FDR to 5%). This results in a list of identified proteins, validated when they had been identified with at least two peptides. Mascot search results were further imported into Progenesis (version 2.2.04), which allows the extraction of ionic currents associated with each peptide. For each experimental population, proteins were submitted to relative quantification using non-conflicting peptides (not found in another protein); the protein abundance in an experimental population being calculated from the sum of non-conflicting peptide ion abundances corresponding to that protein.⁸⁷ From these peptide abundances, we performed ANOVAs to compare the protein expression between low- and high-density populations and calculated fold changes using the protein abundances obtained per experimental population for each protein identified with at least two unique peptides. Finally, we also computed the rank of proteins of each differentially expressed protein in terms of its relative abundance in the averaged pollen proteome, combining results obtained for all experimental populations.

The identified proteins were finally blasted to the TAIR database³⁹ in order to identify the most homologous ortholog in *Arabidopsis thaliana* for functional annotation. We reported the percentage of homology between our protein sequences and the most similar ortholog gene found in the *A. thaliana* database. When two *A. thaliana* orthologs showed similar degrees of orthology (difference in %

homology < 5 points), we retained both orthologs for further functional analysis. Here, we searched the literature using the functional annotation of identified homologous orthologs to seek a functional description of the identified proteins. The same functional characterization was performed for the twelve most abundant proteins identified in the overall expression, combining all experimental populations of *M. annua*.

Quantifying the evolution of pistil morphology

To test our hypothesis regarding pistil evolution in relation to the level of polyandry, we harvested three pistils on several females belonging to the different experimental populations. To avoid introducing variance between plants in pistil morphology due to flower position on the female plant, we harvested pistils on the three first mature flowers along the main branch of each female. Harvested pistils were placed on a microscope slide and positioned to obtain a longitudinal view of the pistil (Figures 1 and S1). A picture of each harvested pistil was taken using a camera coupled to a microscope (EVOS XL Core), maintaining the same 4x magnification to allow for a further comparison of the size of different parts of the pistils between treatments. Pistil trait measurements were sometimes impossible because of the low image resolution or because the view of the pistil was insufficiently longitudinal for fair image comparison. After discarding these pistils, our measured pistil dataset contained between 7 and 17 females (average of 12.5) and between 21 and 50 pistils (average of 36.5) measured per population. In total, our dataset included 125 females and 365 pistils (see Table S3 for population details). Each pistil picture was analyzed using ImageJ.⁶⁹ We measured (a) the style length, (b) the maximal width of one of the stigma lobes, (c) the total length of both the style and one lobe of the stigma, and (d) the length of three randomly chosen stigma hairs (Figure S1).

Statistical analysis of the pollen and pistil phenotypes

To test whether pollen or pistil morphology had diverged over the course of the experiment between the low- and high-density treatments, we compared the likelihood of linear models explaining pollen or pistil traits by the evolutionary treatment (plant density) or not, while taking into account the fact that observations within a given experimental population are not independent (through the structure of random effects). Importantly, experimental populations are recognized as the relevant level of replication in experimental evolution protocols, and statistical analysis need to reflect whether different populations at one evolutionary treatment have converged on a common evolutionary solution that differs from that of populations from another treatment.⁸¹ Specifically, we constructed linear mixed models (LMM) for each of the pollen/pistil traits measured as the response variable, with evolutionary treatment (high- versus low-density populations) declared as a fixed factor. We treated populations (nested within treatment) and individual (nested within population) as random effects to take into account the non-independence of individuals within populations, and of the several pollen/pistils measurements performed on each plant. For pollen traits, we declared two additional random effects: inflorescence (nested within individuals) and an experimental temporal block; this was because several pollen grains were measured from each sampled inflorescence, and because temporal variation in external condition in two rounds of pollen measurements may have affected trait values. To test for an evolutionary response in pollen and pistil traits, we used likelihood ratio tests (LRT) to compare models that included or excluded the evolutionary treatment as a fixed effect. We performed the same procedure as described above for assessing differences in pollen germination rate between treatments, but using generalized linear mixed models with a binomial error distribution. For all pollen traits and for pollen germination probability, we also tested and accounted for the effect of pollen density by including this variable as a fixed effect in each of the models described above. While keeping the treatment as a fixed effect, we also used LRTs to compare models that included or excluded pollen density as a fixed effect. All statistical analyses of pollen and pistil morphology were performed using the lme4 package⁷¹ in R (version 3.6.3, R Project 2020⁷⁰).