LETTER

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Sex-specific volatile compounds influence microarthropod-mediated fertilization of moss

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Sexual reproduction in non-vascular plants requires unicellular free-motile sperm to travel from male to female reproductive structures across the terrestrial landscape[1]. Recent data suggest that microarthropods can disperse sperm in mosses[2]. However, little is known about the chemical communication, if any, that is involved in this interaction or the relative importance of microarthropod dispersal compared to abiotic dispersal agents in mosses. Here we show that tissues of the cosmopolitan moss *Ceratodon purpureus* emit complex volatile scents, similar in chemical diversity to those described in pollination mutualisms between flowering plants and insects, that the chemical composition of *C. purpureus* volatiles are sex-specific, and that moss-dwelling microarthropods are differentially attracted to these sex-specific moss volatile cues. Furthermore, using experimental microcosms, we show that microarthropods significantly increase moss fertilization rates, even in the presence of water spray, highlighting the important role of microarthropod dispersal in contributing to moss mating success. Taken together, our results indicate the presence of a scent-based 'plant–pollinator-like' relationship that has evolved between two of Earth's most ancient terrestrial lineages, mosses and microarthropods.

The origin of bryophytes (mosses, liverworts and hornworts) during the upper Ordovician period represents a notable event in the evolution of life[3,4], leading to the diversification of terrestrial organisms. From a mating systems perspective, the evolution of bryophytes resulted in sexual reproduction partially escaping the aquatic environment. In mosses, sexual reproduction requires free-motile sperm to 'swim' with the aid of water across the terrestrial landscape to fertile females[1,5], a reminder of its aquatic origins. This model of 'swimming sperm' has led to the general view that sperm dispersal among bryophytes is quite limited, with most fertilization occurring within about 10 cm (refs 6, 7). However, recent research using the moss *Bryum argenteum* shows that moss sperm can be dispersed by microarthropods[2], specifically springtails and oribatid mites, which are common inhabitants of moss patches worldwide[8]. This new research builds on earlier, often overlooked work indicating that arthropods may act as ecologically relevant sperm transport vectors[5]. Furthermore, recent data show that moss sperm can be more long-lived and stress tolerant than believed previously[9,10], potentially enabling sperm to survive during long-distance microarthropod dispersal. Although microarthropods are known to use volatile cues in foraging and for communication[11-13], little is known about whether microarthropods may also use chemical cues to facilitate sexual reproduction in mosses.

Here we assess the potential role of moss volatile cues and microarthropods (springtails) in mediating sperm dispersal in *C. purpureus*, a model cosmopolitan moss species with separate sexes. First, to fully capture the suite of possible volatile organic compounds (VOCs) emitted from intact (non-wounded), sexually expressing (gametoecia-producing) male and female plants, we characterized headspace VOCs using two-dimensional gas chromatography–time-of-flight mass spectrometry (GC × GC–TOFMS). We found that for all sampled populations, female plants released a significantly greater number of VOCs than male plants (104.00 ± 9.27 and 29.86 ± 8.21, respectively; $P < 0.0001$; Fig. 1). In addition, analyses of VOC composition revealed significant sex-specific differences (analysis of similarities (ANOSIM): $R = 0.79$, $P = 0.001$, stress value = 3.8; Fig. 2). A surprising diversity of volatile compounds was identified in headspace analysis using our GC × GC–TOFMS approach, and many of these compounds have been identified previously in floral scents of flowering plants[14].

Second, to determine whether springtails were differentially attracted to the observed sex-specific VOC composition, we conducted a series of preference assays using intact (non-wounded) samples of male and female *C. purpureus*. In the first set of preference assays, springtails were given choices between male and female moss samples in Petri dishes, and were found to be significantly more likely to choose intact reproductive female plants over intact reproductive male plants

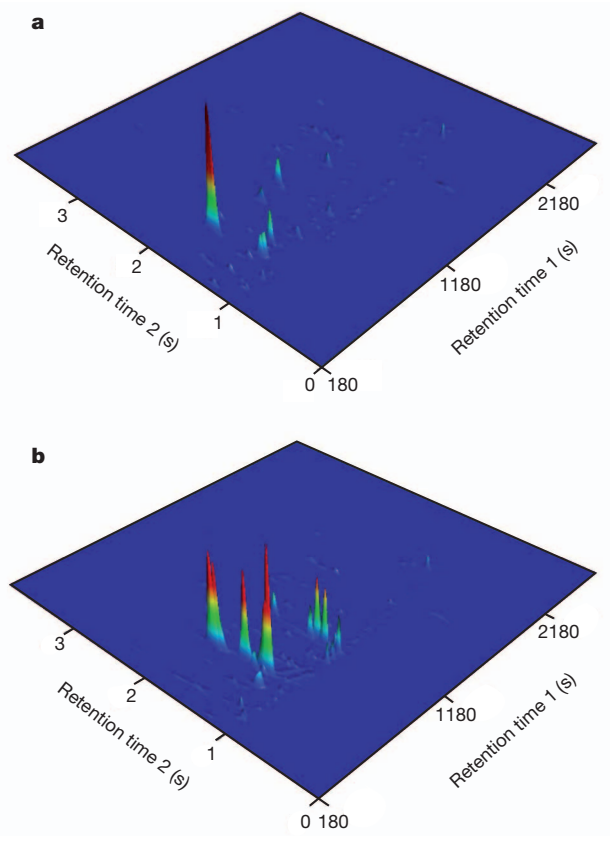

Figure 1 | Sex-specific volatile profiles. **a**, **b**, Representative two-dimensional GC × GC–TOFMS chromatograms of volatile compounds from intact shoots of a reproductive male (**a**) and a reproductive female (**b**) of the cosmopolitan moss *C. purpureus*. Colours indicate relative measures of compound abundance; red indicates compounds that are greater than 50% of the largest individual peak area.

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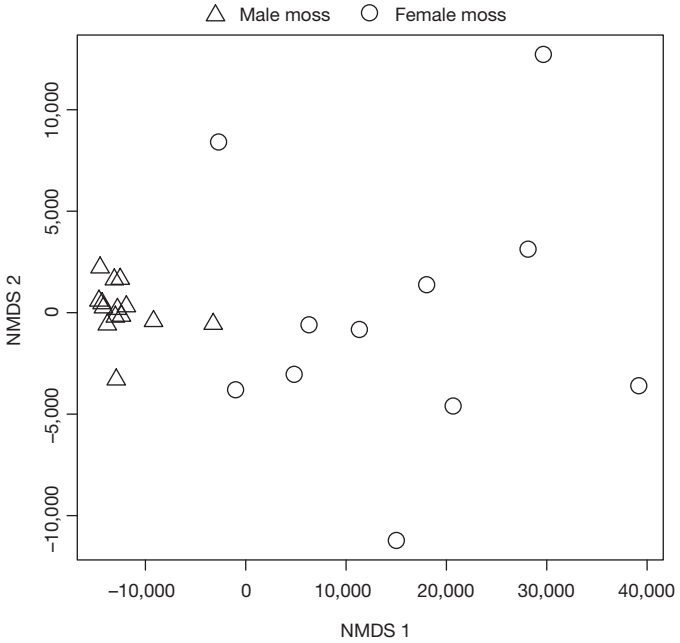

Figure 2 | Differences in volatile composition. Non-metric multidimensional scaling (NMDS) of volatile scent profiles of reproductive male and reproductive female plants of *C. purpureus* shows that there are significant sex-specific differences in VOC composition ($P = 0.001$). Symbols represent scent profiles of individual males and females ($n = 22$, GC × GC–TOFMS analyses).

($G = 37.6$; $P < 0.0001$; Fig. 3). This result is similar to the that of another study[2], in which springtails and mites marginally preferred female to male reproductive *B. argenteum* plants. To confirm that springtail preference for female plants was due to female-specific volatile cues, we used an olfactometer for additional preference assays. Microarthropods were able to assess scents produced by the samples but were not given access to visual or physical cues. In the olfactometer assays, springtails chose intact reproductive female plants significantly more frequently than intact reproductive male plants ($G = 58.1$, $P < 0.0001$). These results reveal the surprising role of volatile cues in influencing microarthropods' choice of intact female moss plants.

Last, we used a series of microcosm experiments in which we manipulated springtail abundance and water spray to assess the importance of biotic versus abiotic factors in promoting sperm dispersal and fertilization in mosses. For this experiment, we used *C. purpureus* and *B. argenteum* (this is the moss species for which springtail-mediated sperm dispersal has been demonstrated previously)[2]. Our results show that for both moss species, the addition of either springtails or water spray significantly increased the number of sporophytes formed per microcosm ($P = 0.05$ and $P = 0.02$, respectively) and the

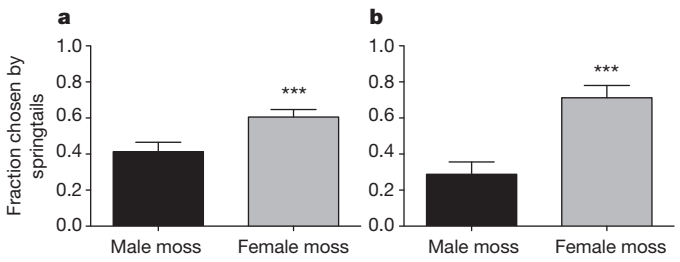

Figure 3 | Springtails prefer female moss. **a**, **b**, The fraction of *C. purpureus* samples chosen by springtails (error bars, mean ± s.e.m.) in preference assays of male versus female samples in Petri dishes (**a**, $n = 24$ assays, 491 springtails counted); and male versus female samples in an olfactometer (**b**, $n = 10$ assays, 276 springtails counted). ***$P < 0.0001$.

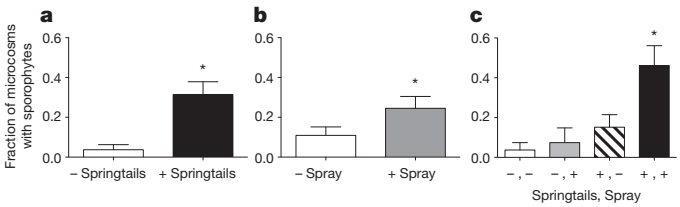

Figure 4 | Springtails enhance fertilization in moss microcosms. **a**–**c**, Fertilization success in *C. purpureus* and *B. argenteum* microcosms, measured as the fraction of microcosms that developed sporophytes (error bars, mean ± s.e.m.). The effects of springtail treatment (**a**), water spray treatment (**b**) and the interaction between these treatments (**c**) on fertilization success. Plus and minus symbols represent the presence and absence of springtails and water spray. $n = 108$ microcosms. *$P < 0.05$.

fraction of microcosms that developed sporophytes ($P = 0.03$ and $P = 0.03$, respectively; Fig. 4). Moreover, the combination of treatments had a pronounced synergistic effect, more than doubling the effect of either treatment alone ($P = 0.03$ for the number of sporophytes per microcosm, and $P = 0.03$ for the fraction of microcosms with sporophytes; Fig. 4c). These results highlight the substantial role of microarthropods in facilitating fertilization in mosses, presumably through enhanced sperm transport.

Plant–insect interactions were key to the diversification of flowering plants[15], with floral scent representing a primary mode of communication between plants and their pollinators[16,17]. Our data suggest that mosses, despite their lack of flowering structures, may similarly utilize volatile scents as cues to manipulate microarthropod behaviour, resulting in increased moss fertilization. Therefore, we propose that there may be a notable plant–pollinator-like relationship that has evolved between microarthropods and mosses involving volatile scent cues.

Sex-specific floral scents have been found in over 20 species of flowering plants with separate sexes, and several ideas have been proposed to explain this pattern[18]. One idea is that the most mate-limited sex is likely to evolve the greatest floral scent[18]. If this theory extends to bryophytes, then our results suggest that female mosses are more mate-limited than males, which is likely given the highly female-biased population sex ratios of these species[19,20], as is typical in mosses[21]. Another idea is that differential pollinator rewards between the sexes may lead to selection for differential cues, including sex-specific VOCs[22]. If, during the normal course of their movements, microarthropods inadvertently pick up released moss sperm from water film[5], or if moss sperm are a food reward for microarthropods (similar to pollen in some plant-pollinator systems), then the reward and cues for male and female moss plants are likely to be different. For example, it has been suggested that females may produce high concentrations of sucrose or fatty acids as a reward[2]. We have not yet distinguished between the composition and amounts of VOCs produced by the reproductive structures and the entire plant, or between the moss tissue and any associated phyllospheric microbes. Sex-specific mutualistic interactions do occur between hosts and microbes[23] and can induce sex-specific VOC differences in the host[24], and it is possible that such interactions may exist in bryophytes. Further studies are needed to establish the fundamental factors in this moss–microarthropod signalling system, including determining which specific VOCs, or suites of VOCs, are most important for signalling as well as pinpointing the cells responsible for the production of key volatile cues. As mosses and microarthropods are two of Earth's most ancient co-occurring terrestrial lineages, it is important to consider the potential role that a plant–pollinator-like relationship may have had in shaping the evolutionary ecology of moss mating systems.

METHODS SUMMARY

To examine volatile profiles in these mosses, gas chromatography was carried out using a Pegasus 4D GC × GC–TOFMS system (LECO). For each sample, 30–40 mg of intact (non-wounded) moss shoots was allowed to equilibrate in a glass vial

for 120 min. Headspace sampling was carried out for 60 min with a solid phase microextraction (SPME) fibre, then thermal desorption of the SPME fibre and analysis by GC × GC-TOFMS was carried out as described previously[25]. Data are based on *C. purpureus* plants collected in Oregon and maintained in greenhouse culture.

To determine springtail preference for male versus female *C. purpureus* samples of intact shoots, we conducted two sets of preference assays. First, for preference assays of whole moss patches, protocols were modified from well-established springtail food preference assays in Petri dishes[26,27], and we used *C. purpureus* plants collected in Oregon and maintained in greenhouse culture. Second, for volatile preference assays, we used a custom-constructed static-air olfactometer designed for springtails[28], and *C. purpureus* plants were collected directly from the field in Oregon. We used two springtail species, *Folsomia candida* and *Sinella curviseta*, for both sets of assays.

To determine the effect of springtails and water spray on moss fertilization, we maintained microcosms of *C. purpureus* ($n = 72$ microcosms) and *B. argenteum* ($n = 36$ microcosms) for approximately 15 weeks in a factorial design with treatments of added springtails and water spray, counting the number of sporophytes after initial sporophyte formation. *C. purpureus* and *B. argenteum* plants were collected in Oregon, Arizona and Kentucky, and maintained in greenhouse culture.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.M.E. and T.N.R. coordinated and planned the project. E.E.S., S.M.E., and T.N.R. initiated and carried out the experiments. A.N.M., J.F.P.. and T.N.R. were responsible for the gas chromatography data and analyses. All authors contributed to the writing of the manuscript.

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METHODS

Study system. *Ceratodon purpureus* (Hedw.) Brid. and *Bryum argenteum* Hedw. are nearly cosmopolitan species, with dioicous breeding systems[29]. For this study, in 2009, we collected plants from three *C. purpureus* populations in the Portland, Oregon metro area from northeast Portland, the Portland State University campus and a farm in North Plains, Oregon, with populations a minimum of 5.8 km apart. The *B. argenteum* plants were collected from 2008 to 2009, from four populations from southern Arizona, the University of Kentucky campus, southwest Portland, Oregon and downtown Portland, Oregon, with populations a minimum of 1.9 km apart. Plants were collected from field populations and grown in the Portland State University greenhouse in pots (6.4 × 6.4 cm) for at least 3 months for all experiments except the olfactometer experiment (for which plants were collected directly from the field). Plants from the *C. purpureus* northeast Portland and Portland State University populations, and the *B. argenteum* Arizona population were grown from spores (single-spore isolations) to ensure that there were separate individuals from these smaller populations, whereas the other plants were grown from single-shoot cuttings, and plants as far apart as possible were collected from within populations. Two commercially available species of springtails, *Folsomia candida* and *Sinella curviseta*, were reared in airtight containers with natural charcoal, de-ionized water and yeast, and were kept in the same growth chambers in which the microcosm experiments were performed (see below). *F. candida* is a model springtail species, growing in soils worldwide[30], and it occurs in high densities in soil and moss communities in the Pacific Northwest region of the Unites States and Canada[31]. *S. curviseta* is an emerging model system as it occurs in sites where *F. candida* is rare[32]. Both species were used in all springtail experiments.

Scent collection and GC × GC–TOFMS analyses. To examine the volatile scent profiles of intact moss tissue, we used static headspace, a method that is sensitive to and can therefore identify small quantities of compounds but cannot be easily used for quantification of amounts of compounds in volatile signatures. For each tissue sample, 30–40 mg of intact (non-wounded) shoots was carefully removed from pots. Each sample was placed into a 2-ml screw-top glass vial and allowed to equilibrate for 120 min. A solid phase micro-extraction (SPME) fibre (polydimethylsiloxane–divinylbenzene, 65 μm coating; Sigma-Aldrich) was then exposed to the headspace for an additional 60 min; results did not change appreciably with additional exposure time (data not shown). Each analysis began by inserting the SPME fibre into the injector (with 'SPME liner') of a two-dimensional gas chromatograph (Pegasus 4D GC × GC–TOFMS system; LECO); the column and analytical conditions used were as described previously for biogenic volatiles[25]. Trace contaminants from ambient air blanks were identified and removed from each of the comprehensive volatile profiles before further data analysis. Comprehensive GC × GC–TOFMS analysis was chosen to minimize any a priori assumptions about the chemical nature of the volatile compounds emitted by the moss system.

To determine whether male and female plants of *C. purpureus* differed in scent composition, we compared overall variation in chemical composition between the sexes, using males and females from each of the three Portland, Oregon populations ($n = 22$ plants in total). For all analyses, we used plants that were producing gametoecia (perichaetia and perigonium, female and male sex organs, respectively, with clusters of modified leaves), and we enriched these structures in the samples. However, initial screens of plant material without gametoecia suggest that male and female plants show a similar difference in volatile composition (data not shown). Further work is required to determine sex-specific ontological and morphological variation in volatile emission rate, site of production and phenological variation. The full list of volatile compounds that we found in the headspace analyses of *C. purpureus* is given in Supplementary Table 1.

Springtail preference assays. To determine whether springtails prefer one reproductive moss sex over the other, and then to test whether this was due to volatile cues, as suggested by the GC × GC–TOFMS analyses, we conducted two sets of preference assays with the moss *C. purpureus*. First, we used protocols modified from well-established springtail food preference assays to construct preference chambers from Petri dishes[26,27], and we conducted preference assays in these dishes comparing male and female intact (non-wounded) reproductive *C. purpureus* samples. For each assay, we used a Petri dish (55 × 15 mm), placed a 55-mm diameter piece of filter paper in the bottom of the dish and placed two smaller pieces of filter paper, separated by 1.5 cm, on top of the larger filter paper. The two comparison samples (5-mm diameter moss patches of intact shoots) were placed on the two smaller filter papers. Moss samples were from two Oregon populations (northeast Portland and Portland State University populations), and both males and females were producing gametoecia. Using a metal spatula, we placed 20 to 40 springtails in each dish between the moss samples, wrapped the dishes in parafilm, darkened them with foil and placed them in the growth chamber in which the springtails were reared. After 120 min, we removed the moss samples and filter paper, and determined the number of springtails within each moss sample, the number of springtails that did not occupy a moss sample and the number of moss shoots and moss reproductive structures per sample. Plants were dried in a drying oven at 60 °C for 48 h and the dry weight was determined. We conducted 24 assays with 491 springtails choosing specific moss samples. Springtails were never reused in assays. We found no significant difference in dry weight between male and female moss samples ($P = 0.95$; mean ± s.e.m. = 8.8 ± 1.2 mg and 9.1 ± 1.3 mg for males and females, respectively). However, male moss samples had significantly more shoots and gametoecia per shoot than did females ($P = 0.03$; mean ± s.e.m. = 25.7 ± 1.88 and 20.3 ± 1.6 for male and female shoots, respectively; $P = 0.001$; mean ± s.e.m. = 1.46 ± 0.32 and 0.22 ± 0.02, for gametoecia per male and female shoot, respectively).

To determine whether the preference that we found for female plants was due to springtails perceiving a volatile cue or another type of assessment (for example, visual), we set up a second set of assays using a static air olfactometer with intact (non-wounded) male and female *C. purpureus* samples. The olfactometer was a modified version of that described in a previous study[33], with the same additional modifications for springtails as described in another paper[28] The olfactometer was made of clear acrylic pipe with two sample compartments divided by a vertical plate. A walking arena for the springtail was placed above the compartments, with the springtails separated from the samples by a wetted opaque filter, to obscure visual choice. For each assay, a male and a female moss sample (15-mm diameter moss patches of intact shoots) were added to separate compartments of the olfactometer. Plants were collected in the field in May 2012 from several sites within a large population (>6,000 m^2) in North Plains, Oregon, and plants were nearing the end of the fertilization season, with females producing a few gametoecia and many new sporophytes, and males producing many gametoecia with ripe antheridia. Using a spatula, we placed 20 to 40 springtails on the walking arena, the olfactometer and placed it in the dark, and we recorded the springtails' choices every 30 min for 120 min. We conducted 10 assays with 276 springtails choosing specific moss samples. We dried and weighed the moss samples, as for the previous assays. We found no significant difference in dry weight or the number of shoots between male and female samples ($P = 0.99$; mean ± s.e.m. = 176.31 ± 18.86 mg and 160.00 ± 29.84 mg for the dry weight of males and females, respectively; $P = 0.53$; mean ± s.e.m. = 0.21 ± 0.03 and 0.21 ± 0.04 for male and female shoots, respectively). Male moss samples differed significantly from female samples in the number of gametoecia per shoot ($P = 0.0003$; mean ± s.e.m. = 0.43 ± 0.07 and 0.21 ± 0.04 for males and females, respectively).

Bryophyte microcosms. To determine the effect of springtails and water spray on sperm dispersal in mosses, we set up factorial experiments in which we manipulated springtail and water spray levels in *C. purpureus* and *B. argenteum*, and we counted sporophyte number as an estimate of fertilization success using a method described previously[34]. To establish microcosms, we propagated the moss on a substrate of a 2:1 mixture of propagation grade sand and peat moss. The mosses were propagated by chopping fresh plant material and distributing the chopped material evenly among microcosms (pots of 6.4 × 6.4 cm), with microcosms containing either *C. purpureus* or *B. argenteum*. For each moss species, microcosms contained plant material from a mix of three to five populations and were composed of both males and females. The microcosms were placed in seedling trays, watered from below and covered in humidomes, to create an enclosed habitat that was conducive to growth for both the springtails and mosses. The experiments were set up in Adaptis 1000 Conviron growth chambers (Pembina), enabling us to control for temperature, light and relative humidity (14 h light–10 h dark cycles with 18 °C light–8 °C dark; 150 μmol of photons m^{-2} s^{-1}; and 65% constant humidity). The microcosms were subjected to one of four treatments: springtails only, water spray only, springtails and water spray, neither springtails nor water spray. Microcosms of water spray and no-spray treatments were evenly distributed among trays of one of two designations (springtails or no springtails). One litre of water was maintained in the base of each tray, and each tray was covered with a humidome lid. Trays were rotated every 2 weeks within growth chambers to control for chamber effects. Water spray was applied approximately once per week with a squirt bottle containing room-temperature spring water. After 80 days in microcosms, an excess of algae accumulated in the *B. argenteum* microcosms, and the spray treatment was intermittent to allow the plants to recover; however, the spray was maintained at least every 14 days. Springtails (approximately 20 per microcosm) were added from stock cultures to all appropriate treatment trays once every 2 to 3 weeks and were observed living in the treatment microcosms in the weeks after application. We counted the number of sporophytes in each treatment after initial sporophyte formation (which we defined as the day when at least 15% of microcosms had sporophytes). The *B. argenteum* was started in August 2010 and took 44 days to reach initial sporophyte formation after planting. The *C. purpureus* were run as two separate experiments (starting in July 2010 and September 2010) with several trays per treatment for each set. One set took 231 days, whereas the second set took 179 days to reach initial sporophyte formation after planting.

We used a third experiment of *C. purpureus* ($n = 32$ microcosms) with the same treatments to test for variation among springtail treatments in the number of gametoecia, chlorophyll fluorescence of photosystem II efficiency (variable:maximum fluorescence (F_v/F_m)); and plant nitrogen content. We found no significant differences among springtail treatments in any of these measures, although adding springtails increased sporophyte production, as in the other experiments. These data suggest that the springtail addition did not enhance reproductive expression leading to more sporophyte production, and it did not alter overall plant health before sporophyte formation, consistent with a role of springtails in mediating sperm transfer.

Data analysis. Multivariate analysis was used to discriminate among volatile scent profiles[35]. Specifically, non-metric multidimensional scaling (NMDS) and analysis of similarities (ANOSIM) were carried out using the R Project for Statistical Computing to test for differences among volatile scent composition between male and female plants. Prior to analyses, individual volatile compounds were sorted into one of 21 IUPAC compounds classes and square-root transformed. All other analyses were conducted using JMP Version 10.0 (ref. 36). To test for differences in the average number of VOCs released between male and female plants, we used *t*-tests. To determine whether springtails chose preferentially between the two samples for each of the two types of preference assays (female versus male samples in Petri dishes or in the olfactometer) at 120 min, we used *G*-tests. We used springtail choice data from the olfactometer assays at 120 min only because there was no significant difference among time points. For preference assays, we used *t*-tests to determine whether male and female moss patch samples differed in dry weight, shoot number or number of gametoecia per shoot. We used logit analysis to determine the effect of the springtail treatment, the water spray treatment and the interaction between these treatments on the fraction of microcosms with sporophytes. We included seedling tray, nested in springtail treatment, in the model. For the logit analysis, we included species and interactions with species but found that these were not significant, and they were therefore dropped from the model. We also used a similar mixed-model nested analysis of variance (ANOVA) to analyse how the number of sporophytes per microcosm (log transformed) were affected by these factors.

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