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LIMITS TO SEXUAL REPRODUCTION IN GEOTHERMAL BRYOPHYTES

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Previous research suggests that while sexual reproduction generally increases with environmental stress it may decrease with extreme stress, at the edge of eukaryotic life. In this study, we explored the limits to sexual reproduction in an extremophile, bryophyte system to ultimately understand the processes that limit sexual reproduction. We used field data from geothermal sites at Lassen Volcanic National Park, California, to demonstrate that sexual reproduction, as measured by the number of sporophytes per shoot, decreases with increasing environmental stress. We found that the number of sporophytes per shoot is positively correlated with distance from geothermal features. When *Pohlia nutans* plants were transplanted to mesic conditions, high numbers of gametocidia and sporophytes were produced, regardless of where along the environmental stress gradient plants originated, suggesting that physiological stress rather than local adaptation is constraining sexual reproduction in this extremophile system. We discuss our results with respect to previous work on sex in extreme environments.

Keywords: bryophyte, reproduction, stress, temperature.

Introduction

Understanding the processes controlling the evolution and maintenance of sex is a key unresolved problem in evolutionary biology (Smith 1978; Burt 2000, 2002; Vamosi et al. 2003). While theoretical and laboratory-based studies suggest that sexual reproduction is favored in stressful environments (Iglesias and Bell 1989; Zeyl and Bell 1997; Greig et al. 1998; Grishkan et al. 2002), few studies have experimentally examined the relationship between environmental stress (either biotic or abiotic) and sexual reproduction in natural systems (e.g., Lively et al. 1998; West et al. 2002; Kis-Papo et al. 2003; Nedelcu and Michod 2003; Nedelcu et al. 2004) and fewer still have sought to determine what limits sexual reproduction in extremophiles living at the edge of eukaryotic life.

Recent research has rapidly advanced our understanding of the theoretical parameters underlying constraints on the evolution and maintenance of sex (e.g., Otto and Michalakis 1998; West et al. 1999; Vamosi et al. 2003); however, empirical work on biological constraints to sexual reproduction in stressful environments lags far behind these recent theoretical gains, mainly due to the lack of tractable experimental systems (Nedelcu and Michod 2003; David et al. 2005; Killick et al. 2006). Theory suggests that rates of sexual reproduction might increase in stressful, inhospitable, and crowded conditions because sex allows, among other things, (1) the production of offspring with allele combinations that may be more fit than the parental genotypes (see Burt 2000 for review) and (2) the operation of DNA repair mechanisms (e.g., Nedelcu and Michod 2003). If, in fact, sexual reproduction is beneficial, then we should find evidence of mechanisms

facilitating successful sexual reproduction under high-stress conditions. Likewise, the need to identify the physiological limitations to sexual reproduction during extreme stress is necessary in order to distinguish whether limits to sexual reproduction are a consequence of physiological constraints on reproduction or instead reflect a selective optimum because sexual reproduction is disadvantageous in extreme stress environments (Kis-Papo et al. 2003).

In this study, our aim is to explore the limits to sexual reproduction in a geothermal bryophyte system. Bryophytes colonize the hottest terrestrial sites in geothermal areas worldwide, and these sites have varying moisture levels (Given 1980; Kappen and Smith 1980; Smith 1981; Bargagli et al. 1996; Burns 1997; Glime and Hong 1997; Elmarsdottir et al. 2003; Bonini et al. 2005; Convey and Lewis Smith 2006). Bryophytes are ecologically ubiquitous, occupying habitats on every continent and in nearly every terrestrial ecosystem (Shaw and Goffinet 2000). Bryophytes withstand extreme desiccation (Dilks and Proctor 1979), live at extreme temperatures (Given 1980; Burns 1997), and reproduce both sexually and asexually (Wyatt and Anderson 1984), making these organisms an ideal model system for examining the relationship between ecological stress and sexual reproduction. In addition, the presence of sporophytes is a well-established “marker” for realized sexual reproduction (i.e., successful fertilization) in bryophytes, making assessment of successful sexual reproduction feasible under field conditions (Mishler 1990). Despite obvious and intriguing experimental advantages, relatively few studies have examined the ecological or physiological limits to sexual reproduction in bryophyte systems (Shaw 2000).

Here we present research examining how patterns of sexual reproduction vary across an extreme environmental gradient within a well-characterized geothermal bryophyte community at Lassen Volcanic National Park (LVNP), California. We con-

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ducted extensive surveys of geothermal moss populations at LVNP surveying locations in which midday temperatures at the rhizoid-soil interface ranged from 15° to 59°C and use this system to address these questions: (1) does sexual reproduction, as measured by the number of sporophytes per shoot, increase with environmental stress as has been shown for other eukaryotes; (2) is the number of sporophytes per shoot correlated with either sexual expression or sex ratio of reproductive structures in geothermal bryophytes; and (3) does transfer of high-stress individuals to nonstress conditions alter the pattern of sexual reproduction in geothermal bryophytes? To answer these questions, we surveyed sexual structures in bryophytes in geothermal sites at LVNP and transferred plants from the field to the greenhouse to assess the role of local environment in constraining components of sexual reproduction in geothermal bryophytes.

Material and Methods

Study System

We have extensively surveyed for bryophytes around geothermal features in LVNP. Surveys in 2006 found bryophyte communities at Boiling Springs Lake (40.435695°N, -121.397145°W; mean elevation of 1799 m) and Devil's Kitchen (40.44164°N, -121.434331°W; mean elevation of 1854 m) in the southeastern edge of the park but not at eight additional geothermal regions in the park where surveying was feasible. The chemical composition of these springs and geological systems of the areas has been well documented (Thompson 1982, 1983; Muffler et al. 1983; Synder 2005; Siering et al. 2006). At the elevation of these springs, the nongeothermal areas are primarily mixed coniferous forests of *Pinus jeffreyi* Balf. and *Abies concolor* (Gord. & Glend.) Lindl. ex Hildebr., with few bryophytes except occasional *Polytrichum juniperinum* Hedw. patches in forest gaps. Besides plant communities in geothermal areas, the only nonconiferous communities in this region of the park are wet meadows (Pinder et al. 1997), and these have extensive bryophyte communities dominated by *Philonotis fontana* (Hedw.) Brid. and *Brachythecium frigidum* (Müll. Hal.) Besch.

From surveys in 2006, 2007, and 2008 at Boiling Springs Lake and Devil's Kitchen, we identified four primary bryophyte species growing in geothermally heated soils: *Aulacomnium palustre* (Hedw.) Schwägr., *Campylopus introflexus* (Hedw.) Brid., *Ceratodon purpureus* (Hedw.) Brid., and *Pohlia nutans* (Hedw.) Lindb., which all have been reported in geothermal sites in other areas of the world (Scott and Stone 1976; Gradstein and Sipman 1978; Given 1980; Söderström 1992; Bargagli et al. 1996; Burns 1997; Glime and Hong 1997; O'Brien 2000; Skotnicki et al. 2001,2002; Elmarsdotir et al. 2003; Smith 2005; Convey and Lewis Smith 2006; Chiarucci et al. 2008). In LVNP, *Aulacomnium palustre* grows exclusively around Boiling Springs Lake, and *Pohlia nutans* grows exclusively around Devil's Kitchen. Also, the bryophytes *Bucklandiella affinis* (Schleich. ex Weber & Mohr) Bednarek-Ochyra & Ochyra and *Ptychostomum pseudotriquetrum* (Hedw.) J.R. Spence & H.P. Ramsay occur in geothermal areas around Boiling Springs Lake but in fewer populations than the other bryophyte species. Of the six

bryophytes growing in geothermally heated soils at Lassen, five have separate sexes, and *P. nutans* is hermaphroditic (Watson and Richards 1968; Crum 1973; Scott and Stone 1976). Just outside of the thermally heated areas, but in areas where the forest canopy remains open, *Aulacomnium androgynum* (Hedw.) Schwägr., *Bucklandiella occidentalis* (Renauld & Cardot) Bednarek-Ochyra & Ochyra, *Dicranum tauricum* Sapeh., and *Grimmia montana* Bruch & Schimp occur. Thus, we have found 10 bryophyte species in and around these geothermal springs at LVNP. Angiosperms associated with the geothermal areas at LVNP include *Dichanthelium acuminatum* (Sw.) Gould & C.A. Clark, *Agrostis scabra* Willd., and *Mimulus guttatus* DC.

Sporophyte Survey 2006

To determine the extent of sexual reproduction in bryophytes around geothermal features in LVNP, we sampled bryophytes in the field and counted sporophytes on the bryophytes we brought back to the lab. Sporophyte counts (including current sporophytes and indications of past sporophytes) maximize detection of sexual reproduction patterns in bryophytes (Stark et al. 2005) and have been used previously as an accurate indicator of frequency of realized sexual reproduction in bryophytes (Mishler 1990). In August 2006, we sampled bryophytes along nine line transects at Devil's Kitchen and four line transects at Boiling Springs Lake, with transects beginning near geothermal springs and fumaroles and ending farther away from geothermal features. We placed transects in all areas that we could safely reach in 2006. Depending on the size of bryophyte communities around each geothermal feature, transects were either 270 cm, with 2.5 × 2.5-cm bryophyte mats collected every 30 cm, or 100 cm, with 2.5 × 2.5-cm bryophyte mats collected every 20 cm. Temperature was measured at each mat collection location using a HH603A thermocouple with a 3-mm-diameter copper-constantan probe (Omega, Stamford, CT) placed at the interface between the moss rhizoids and the soil without rhizoids, generally a distinct boundary in the geothermal species. Measurement times for temperatures varied ±2 hours from midday.

The 2.5 × 2.5-cm bryophyte mat was our sampling unit for this survey and the subsequent survey in 2007. Bryophyte mats were collected, dried, and brought back to the lab at Portland State University. Bryophyte mats were rehydrated, the number of species was recorded, the species were identified, and the number of sporophytes (diploid tissue after fertilization) was counted on 300 gametophytic shoots per species, unless fewer shoots were available, in which case the total number of sporophytes on all shoots was counted. Gametophytic shoots within a bryophyte mat are expected to often represent individuals from the same genet, but some genetic diversity is possible (i.e., Van der Velde et al. 2001). Sporophyte counts included current cycle sporophytes and evidence of previous cycle sporophytes, following Stark et al. (2005). For the surveyed transects at Lassen in 2006, we surveyed eight bryophyte species (*A. palustre*, *C. introflexus*, *C. purpureus*, and *P. nutans* in the hotter areas, and *A. androgynum*, *B. occidentalis*, *D. tauricum*, and *G. montana* in the cooler sites), and we surveyed a mean of 279.3 ± 5.90 (SE) shoots per bryophyte mat collected.

*Sexual Expression, Gametoezia Sex Ratios,
and Sporophytes 2007*

To determine whether the low number of sporophytes we found in geothermal sites in 2006 was potentially caused by either (1) low production of sexual structures by gametophytes or (2) a skewed gametoezia sex ratio, we examined the number of sporophytes per shoot and the number and sex of gametoezia (perichaetia and perigonia, female and male sex organs, respectively, with clusters of modified leaves) per shoot in 2007. We examined individuals from populations of *A. palustre*, *C. purpureus*, *C. introflexus*, *P. pseudotriquetrum*, *P. nutans*, and *B. affinis* for gametoezia and sporophytes. We chose *P. pseudotriquetrum* and *B. affinis* despite these samples not appearing in our 2006 survey, because changes in the geothermal areas allowed us safe access to these species, and these species occurred in the hotter sites, growing with *C. introflexus*. For species with separate sexes, shoots were expected to have either male or female gametoezia, and we calculated the gametoezia sex ratio as the fraction of male gametoezia (male gametoezia divided by the total number of gametoezia). *Pohlia nutans*, the hermaphroditic species, could potentially have both male and female gametoezia on the same plant, and we calculated the gametoezia sex ratio in the same manner as with dioecious species (male gametoezia divided by the total number of gametoezia). Populations were defined for this study as discrete patches of a bryophyte species that varied in distance from one another from 20 m to more than 5 km within the Lassen geothermal area; two or three populations were sampled per species except for *B. affinis*, for which only one population is present at LVNP. Thus, in 2007, we had more exhaustive coverage from more populations of the most common species at LVNP. For each population, transects were either 270 cm, with bryophyte samples collected every 30 cm, or 100 cm, with bryophyte samples collected every 20 cm, with the first sample of each transect taken in the moss patch closest to the geothermal site, as in 2006. Larger populations included more than one transect, separated by 100 cm. We collected 2.5 × 2.5-cm bryophyte mats for each bryophyte sample, as previously. Samples were air-dried for storage until they were processed. Bryophyte mats were examined under a dissecting scope to determine the number of reproductive structures (gametoezia as well as counts of current and prior sporophytes) per shoot; 50 shoots were examined unless fewer shoots were present in a location, in which case the total number of reproductive structures on all shoots was counted (we examined 4744 shoots; 570–1126 shoots per species).

Common Garden Experiment with P. nutans

To determine whether the low number of sporophytes along geothermal gradients was due to local adaptation or to physiological constraint under extreme stress, we collected *P. nutans* plants from along the geothermal gradient and grew them in a common garden environment in a greenhouse at Portland State University. We used *P. nutans* because it has a hermaphroditic sexual system, and thus, issues of sex ratio bias (i.e., too few males to produce sporophytes) will not confound our analysis. We used plant material collected from

our 2007 transects with *P. nutans* (see above), and thus, information on the location of collected plants relative to geothermal features was recorded. The numbers of gametoezia and sporophyte per shoot were also recorded in our 2007 transect counts, and these data were used to compare the number of reproductive structures per shoot on *P. nutans* plants in the field to *P. nutans* plants acclimatized to growing in the greenhouse (see below). Fourteen sampled mats with *P. nutans* from three populations were randomly chosen for transfer to the greenhouse. Sample sizes were limited for this experiment because our park permit restricted us from collecting and maintaining larger collections of plants from these fragile geothermal habitats.

From the 14 randomly chosen 2.5 × 2.5-cm *P. nutans* samples from the 2007 transects, we removed 20 shoots and planted them into two pots (10 shoots per pot) in the greenhouse (15° ± 1.5°C; automatic misting watering system with twice-daily watering for 1 min per cycle). We allowed the shoots to reproduce through the protonemal stage to reduce variation due to previous environmental variation (Shaw 1986). Plants remained in the cultures for 2 yr. In the first year, mature sporophytes were removed to prevent colonization among pots.

In the second year, for the 12 pots from two populations that survived transplantation in the greenhouse, we recorded the numbers of gametoezia and sporophytes per shoot. We counted shoots randomly within pots until we recorded 50 nonexpressing shoots, and we recorded the number of expressing shoots (gametoezia) and shoots with sporophytes within those samples. On average 182.82 ± 13.09 (SE) shoots were sampled in each pot for this analysis (50 nonexpressing shoots and the remainder with gametoezia or sporophytes). We used this method rather than a determined number of shoots because plants had several sexual stages (gametoezia and sporophytes) simultaneously and differed in the mix of these stages; *P. nutans* goes through multiple waves of reproduction (Clarke and Greene 1971), resulting in gametoezia and sporophytes overlapping.

Data Analysis

For the 2006 data, we used regressions to determine whether the number of sporophytes per shoots varied with (1) distance from geothermal features and (2) temperature at the rhizoid-soil interface across all species, rather than within species, as species distributions and frequencies within sites were not large. We did use generalized linear models (GLMs) to explore the effects of transect, population, species, site (Devil's Kitchen or Boiling Springs Lake), and interactions with these effects on our analyses, but we found they had no effect, perhaps because our samples sizes within species were not sufficiently large. For *P. nutans*, the only species that occurred at higher frequency across sampling locations, we used a one-way Welch's ANOVA to test whether sampled mats had more sporophytes per shoot in geothermal sampling locations rather than mats in nongeothermal sampling locations. Geothermal sampling locations (G) were determined to be those locations with active, steaming hydrothermal fissures and were within 60 cm of geothermal features (pools or fumaroles). Nongeothermal sampling locations (NG) were all

Table 1

Bryophyte Species Occurring within Geothermal Sites in Lassen Volcanic National Park and Midday Temperatures Measured at the Rhizoid-Soil Interface during Our 2006 Sporophyte Survey

Species	<i>n</i>	Mean temperature (°C) ± SE	Temperature range (°C)
<i>Bucklandiella occidentalis</i>	3	19.96 ± .26	19.50–20.39
<i>Grimmia montana</i>	2	23.38 ± 2.83	20.55–26.22
<i>Aulacomnium androgynum</i>	2	23.94 ± 5.61	18.33–29.55
<i>Ceratodon purpureus</i>	11	26.92 ± 3.78	17.83–48.67
<i>Dicranum tauricum</i>	2	28.67 ± .22	28.44–28.88
<i>Poblia nutans</i>	66	29.02 ± .60	19.83–54.55
<i>Aulacomnium palustre</i>	20	39.10 ± 2.93	15.61–57.89
<i>Campylopus introflexus</i>	10	43.94 ± 2.63	30.33–59.44

Note. Species are listed from low to high mean temperature.

other locations on the sampled transects. We chose a Welch's ANOVA because of heterogeneity of variances in our data (Day and Quinn 1989).

For the 2007 data, we had larger sample sizes within species, and thus we used a generalized linear model to determine the effect of transect (nested in species), species, distance from geothermal features along transects (a continuous variable), the number of gametocidia (a continuous variable), and interactions among these factors on the number of sporophytes per shoot (a continuous variable), for the three species in which we recorded both sporophytes and gametocidia present during the survey. Site (Devil's Kitchen or Boiling Springs Lake) was not significant and was not included in the analyses. We used a similar model to determine the effect of transect (nested in species), species, gametocidia sex ratio (a continuous variable), and interactions among factors, on the number of sporophytes per shoot for the subset of samples for which we had males and/or females present. No interactions were significant, and they were dropped from the analysis. We ran this model with and without *P. nutans*, which has combined sexes and thus is an outlier compared with the other moss species which all have separate sexes. However, we found no qualitative difference when we included *P. nutans* in the model compared to when it was excluded (these data are not shown). There were not sufficient degrees of freedom to include both transect and population in the models; as our sampling design was based on transect, we included transect and not population, although the models were qualitatively similar with either factor included.

In a comparison of plants grown in the greenhouse and field, initial ANOVA analyses determined that transect was not a significant factor. Because of heterogeneity of variance in analyzing our data, we dropped transect from our models and used Welch's ANOVA to determine the effect of growth environment (greenhouse vs. field) on (1) the number of gametocidia per shoot and (2) the number of sporophytes per shoot in *P. nutans* plants. For these analyses, we used field data from *P. nutans* plants collected during 2007, which were from the same populations for which we had greenhouse-grown plants for comparison. We used mixed-model ANOVA to determine the effect of distance of sampling location from geothermal features (a fixed effect and a categorical distance variable coded as either geothermal [G] or nongeothermal [NG], as above described above for the *P. nutans* in the 2006 survey) and transect (a random effect) on (1) the

number of gametocidia per shoot and (2) the number of sporophytes per shoot in *P. nutans* grown in the greenhouse experiment. All analyses were performed using JMP 8.0.2 (SAS Institute 2009).

Results

Distribution and Sporophyte Survey 2006

Aulacomnium palustre, *Ceratodon purpureus*, and *Poblia nutans* occurred most frequently in our survey, and occurred in a range of soil temperatures. *Campylopus introflexus* occurred frequently in our survey but only in high-temperature

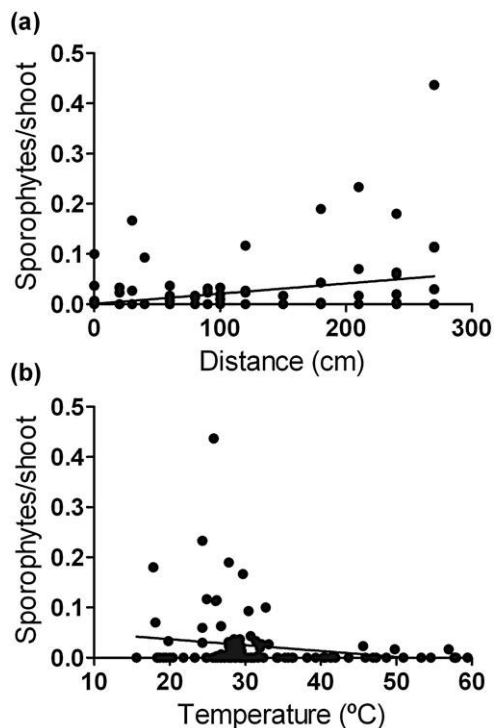


Fig. 1 Mean (\pm SE) sporophytes per shoot versus distance from geothermal features (a) and temperature at the rhizoid-soil interface (b) for bryophytes sampled at Lassen Volcanic National Park in 2006; $n = 108$ mats sampled.

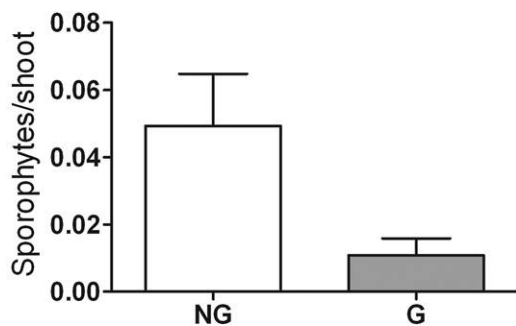


Fig. 2 Mean (\pm SE) sporophytes per shoot for the moss *Pohlia nutans* from geothermal (G) and nongeothermal (NG) locations in which the moss occurred in the 2006 survey; $n = 35$ mats sampled in nongeothermal locations and 31 mats sampled in geothermal locations.

soils (table 1). *Aulacomnium androgynum*, *Bucklandiella occidentalis*, *Dicranum tauricum*, and *Grimmia montana* occurred rarely in open areas farther from geothermal features, in soils or on rocks that were less hot. *Ptychostomum pseudotriquetrum* and *Bucklandiella affinis*, two geothermal bryophyte species at LVNP, occurred less frequently in high-temperature soils and were absent from this survey in 2006.

Sporophytes were found in five moss species, *A. palustre*, *C. purpureus*, *D. tauricum*, *G. montana*, and *P. nutans*. The number of sporophytes per shoot increased significantly with distance from geothermal sites across LVNP bryophyte species in 2006 ($n = 108$ mats sampled; $P = 0.002$; $R^2 = 0.09$; fig. 1). Similarly, the number of sporophytes per shoot was significantly influenced by temperature at the rhizoid-soil interface across species ($n = 108$ mats sampled; $P = 0.04$; $R^2 = 0.04$; fig. 2), with a sharp cutoff around 35 °C, above which few sporophytes were found. In *Pohlia nutans*, the one species to occur across many sampling locations in this survey, the number of sporophytes per shoot was significantly higher in nongeothermal sampling locations than in geothermal locations ($n = 66$; $F_{1,40} = 5.61$; $P = 0.02$; fig. 2).

Sexual Expression, Gametoezia Sex Ratios, and Sporophytes 2007

For the six bryophyte species we examined more extensively in 2007, sporophytes and gametoezia were found in three species, *C. purpureus*, *P. nutans*, and *P. pseudotriquetrum*, and gametoezia only were found on *A. palustre* and *C. introflexus*. *Bucklandiella affinis* plants were found to have neither sporophytes nor gametoezia. For the three species with both sporophytes and gametoezia, the number of sporophytes per shoot was significantly affected by distance from geothermal features, the number of gametoezia per shoot, and interactions between these two factors, as well as with species (table 2). Most importantly, the number of sporophytes per shoot was significantly affected by the interaction between distance and the number of gametoezia per shoot, with an increase in the number of sporophytes per shoot with an increase in the distance from geothermal features and an increase in the number of gametoezia per shoot. However, this relationship varied in strength among the three species, as the three-way interaction with species, distance, and gametoezia per shoot was significant in the analysis (table 2). In a separate analysis, which included only samples with expressing males and/or females, we found that the number of sporophytes per shoot was not affected by gametoezia sex ratio ($n = 60$ mats sampled, $P = 0.74$, $df = 1$, $\chi^2 = 0.11$), although transect ($P = 0.02$, $df = 8$, $\chi^2 = 18.77$) and species ($P = 0.006$, $df = 4$, $\chi^2 = 14.31$) were significant in this analysis. Despite a measurable number of gametoezia per shoot in the field in most species (fig. 3a), there was little to no variation in gametoezia sex ratio within species (fig. 3b).

Sexual Expression in the Greenhouse

Transfer of *P. nutans* plants from the field to the greenhouse significantly increased the number of gametoezia and sporophytes per shoot ($n = 35$ pots and bryophyte mats; $P = 0.0004$; $F_{1,32} = 15.49$; $P < 0.0001$; $F_{1,21} = 31.32$; respectively; fig. 4a). Neither distance from a geothermal feature nor transect of the original *P. nutans* sample had a significant effect on the number of gametoezia or sporophytes

Table 2

Results of a Generalized Linear Model Examining the Effect of Transect (Nested in Species), Species, Distance from a Geothermal Feature, Number of Gametoezia per Shoot, and Significant Interactions between Factors on the Number of Sporophytes per Shoot for *Ceratodon purpureus*, *Pohlia nutans*, and *Ptychostomum pseudotriquetrum*

Source	df	χ^2	P
Transect (species)	6	9.65	.14
Species	2	.77	.69
Distance	1	16.79	<.0001
Gametoezia/shoot	1	16.29	<.0001
Species \times distance	2	27.60	<.0001
Distance \times gametoezia/shoot	1	23.21	<.0001
Species \times gametoezia/shoot	2	18.16	<.0001
Transect (species) \times distance	6	23.19	.0007
Species \times distance \times gametoezia/shoot	2	38.21	<.0001

Note. The three bryophyte species surveyed at Lassen Volcanic National Park had both sporophytes and gametoezia in 2007. $n = 55$ bryophyte mats sampled. Significant P values are underlined.

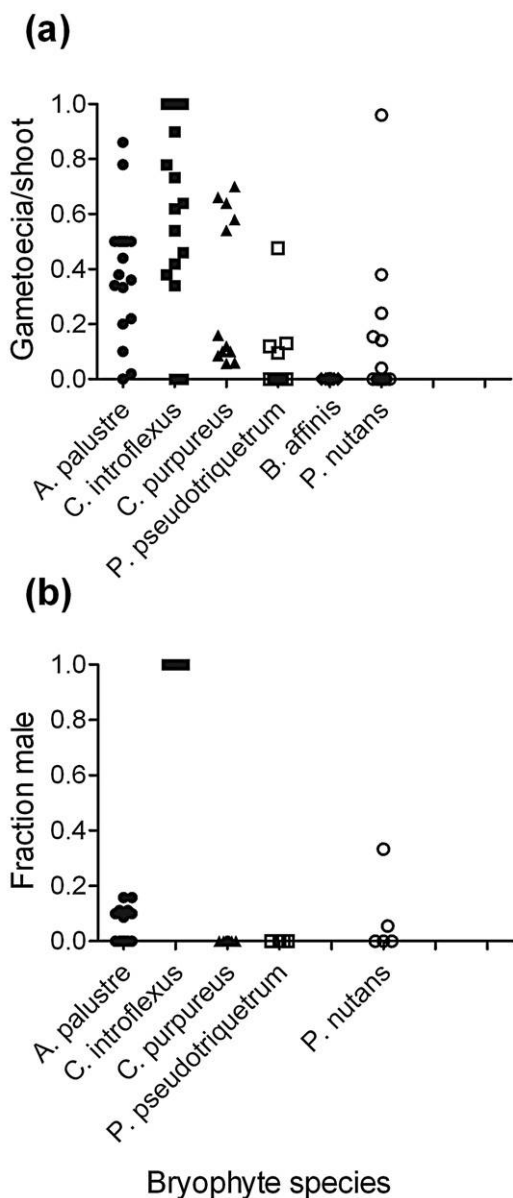


Fig. 3 a, Mean (\pm SE) gametocia per shoots for geothermal bryophytes at Lassen Volcanic National Park (LVNP) in 2007; $n = 107$ mats sampled. b, Fraction of shoots with gametocia that are male for geothermal bryophytes at LVNP; $n = 60$ mats sampled. *Aulacomnium palustre* (closed circles), *Campylopus introflexus* (closed squares), *Ceratodon purpureus* (closed triangles), *Ptychostomum pseudotriquetrum* (open squares), *Bucklandiella affinis* (closed diamonds), and *Poblia nutans* (open circles).

per shoot for plants grown in the greenhouse (table 3; fig. 4b). This contrasts to field-collected *P. nutans* samples in which distance from a geothermal feature had a significant effect on the number of sporophytes per shoot (table 2; fig. 4c), and the number of sporophytes per shoot was significantly affected by the interaction between the number of gametocia per shoot and distance from geothermal features (table 2).

Discussion

In geothermal springs at LVNP, we found that while moss gametophytes are common around many springs in the area, the number of sporophytes is low near geothermal features and increases with distance away from such features and as temperature decreases. To determine at what stage sexual reproduction is being impacted in these geothermal sites, we collected data on both sex expression and sporophyte abundance across the LVNP gradients. Our data suggest that sporophyte production across these species was significantly affected by gameto-

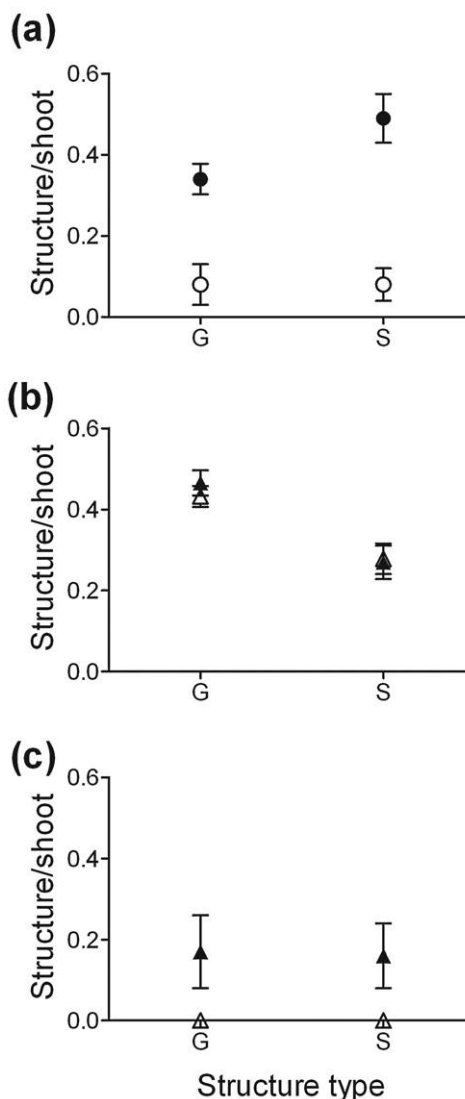


Fig. 4 Mean (\pm SE) gametocia per shoot (G) and sporophytes per shoot (S) for *Poblia nutans* plants. a, Shoots grown in the greenhouse (filled circles) and shoots collected directly from the field (open circles); $n = 35$ samples (12 pots from the greenhouse and 23 sampled mats from the field). b, Shoots grown in the greenhouse but originally collected from geothermal sampling locations (filled triangles) and from non-geothermal sampling locations (open triangles); $n = 12$ pots. c, Shoots collected directly from the field from geothermal sites (filled triangles) and from non-geothermal sites (open triangles); $n = 23$ sampled mats.

Table 3

Results of Two-Way ANOVA Examining the Effect of Transect and Original Collection Distance from Geothermal Features (within 60 cm of Geothermal Features versus Farther from Geothermal Features)

Source	No. gametoezia per shoot			No. sporophytes per shoot		
	df	F	P	df	F	P
Transect	1	.04	.85	1	.32	.59
Distance	1	3.11	.11	1	.04	.85

Note. Data are for *Poblia nutans* from Lassen Volcanic National Park growing in mesic conditions in the greenhouse at Portland State University. $n = 12$ pots. There are no significant P values.

ecia production, and thus in extreme-stress sites, few plants produced either sporophytes or gametoezia (fig. 4c). On transfer to more mesic greenhouse conditions, the correlation between the number of sporophytes and distance from geothermal feature disappears (fig. 4b), at least in *Poblia nutans*, suggesting that variation in the number of sporophytes among plants from the field is due to a physiological response rather than local adaptation. Below, we consider the correlation between sexual reproduction and environmental stress in this system.

Sex and Geothermal Stress

Theoretical and laboratory-based studies across a broad range of taxa demonstrate that sexual reproduction is favored in stressful environments (Iglesias and Bell 1989; Zeyl and Bell 1997; Greig et al. 1998; Grishkan et al. 2002). However, here we show that within geothermal bryophytes at LVNP the number of sporophytes per shoot increases with distance away from geothermal features and decreasing temperature (fig. 1a; table 2), suggesting that bryophytes are undergoing little sexual reproduction within the most extreme geothermal sites. Similar results have been found in a handful of other studies. For instance, Kis-Papo et al. (2003) showed sexual reproduction in fungi was severely reduced in the extreme salinity of the Dead Sea but increased with more moderate levels of stress (Kis-Papo et al. 2003). In mosses, Huttunen (2003) found that *Pleurozium schreberi* and *P. nutans* decreased sporophyte production as stress increased to extreme levels due to copper pollution. Also in mosses, Stark et al. (2005) found that in the extreme stress of the desert, *Syntrichia caninervis* decreased sporophyte production as stress increased. Our data from LVNP geothermal bryophytes supports these studies, indicating that sexual reproduction may decrease under extreme stress.

Temperature is negatively associated with sporophyte number in our 2006 data, suggesting that temperature may be a driver for the negative association between geothermal sites and sporophyte production in the LVNP bryophyte system. However, the R^2 value in our regression of sporophyte production and temperature was not high. Thus, other stresses imposed by the geothermal features, such as heavy metals in the soil, are potentially aiding in the decline in bryophytes and sexual reproduction found near geothermal features at LVNP.

Gametoezia Expression and Sex Ratios

Our data suggest that there is variation in gametoezia expression but not variation in gametoezia sex ratio across the

geothermal stress gradient in the LVNP bryophytes. Our data show that the number of sporophytes per shoot is affected by an interaction between the number of gametoezia per shoot and distance from geothermal features (table 2), suggesting that the variation in the number of sporophytes per shoot with distance from geothermal features we observed at LVNP may be driven by variation in gametoezia formation along this same gradient. However, we also found that the number of sporophytes per shoot is significantly affected by a three-way interaction among species, distance, and the number of gametoezia per shoot. This three-way interaction indicates that for the three species measured, gametoezia expression may be behaving differentially across the geothermal stress gradient. For *Ceratodon purpureus* and *P. nutans*, gametoezia expression increases with distance away from the geothermal features, and this correlates with the number of sporophytes per shoot, but the majority of the sexual expression in *C. purpureus* occurs between 30 and 60 cm from geothermal features. For *P. nutans*, sexual expression occurs exclusively between 100 and 200 cm from geothermal features. This difference between the two species is most likely due to the heat tolerance and distributional variation. *Ptychostomum pseudotriquetrum*, the third bryophyte species in this analysis, has fewer gametoezia overall and a more even distribution of gametoezia across the geothermal stress gradient. Our data on variation in gametoezia expression across the geothermal stress gradient contradict previous data for *P. nutans* showing that reproductive shoots did not vary with distance from copper smelters, also presumably high-stress environments (Huttunen 2003).

For gametoezia sex ratios, we did find that the majority of species we examined had extremely female-biased sex ratios (four out of five species; fig. 3b), suggesting female-biased population sex ratios. However, we found little variation for gametoezia sex ratio within any of the species in geothermal areas across the gradient we sampled. These data suggest that while males and females may be differentially affected by stress, this differential did not change across the stress gradient, and differences in gametoezia sex ratio are not responsible for the variation in sporophyte production we measured across the gradient.

Mechanisms for Limits on Sexual Reproduction

Sexual reproduction may be limited in extreme environments either because (1) physiological constraints make sexual reproduction difficult or (2) only a few adaptive genotypes are

selected as a selective optimum is reached and thus sexual reproduction is disadvantageous (Kis-Papo et al. 2003). Determining whether physiology or adaptation is responsible for the lack of sporophytes we find near geothermal features in geothermal bryophytes will be important to understanding sexual reproduction in extreme-stress systems and for potentially understanding the evolution of sex in a range of systems that may have evolved under extreme stress. We believe our data from *P. nutans* hint at what limits sexual reproduction in the geothermal bryophytes at LVNP. When we grow *P. nutans* plants from across the extreme stress gradient in mesic greenhouse conditions, plants produced both more gametocidia and sporophytes per shoot than were observed in natural populations of *P. nutans* growing across the geothermal gradient (fig. 4a). Further, acclimatizing plants to greenhouse conditions allowed them to produce gametocidia and sporophytes regardless of whether they were originally growing in high-temperature locations near geothermal features or in low-temperature locations away from geothermal features (fig. 4b). In particular, despite the low sample size, the fact that plants from near geothermal areas produced gametocidia and sporophytes in the greenhouse when they had not in the field suggests that they are physiologically able to do so and are not genetically restricted from such production. These data differ from those of Jules and Shaw (1994), who transferred *Ceratodon purpureus* individuals from a high heavy-metal area to soil from low heavy-metal areas and did not see increases in gametocidia. Though not conclusive, our data do suggest that physiological constraints rather than local adaptation are limiting sexual reproduction in this system.

When plants are released from the constraints imposed by extreme stress they may reproduce sexually with little apparent trouble, as *P. nutans* did in our greenhouse experiment. It is possible that plants from the geothermal areas are simply adapted to be more plastic than plants from nongeothermal areas at LVNP, and a fully reciprocal experiment in growth chambers or the field is needed to determine whether this is the case. Field reciprocal transplants would be ideal but are not allowed within geothermal areas in the National Park and have been rarely attempted in mosses (e.g., Kooijman

1993; Frego and Carleton 1995; Mulligan and Gignac 2001; Cole et al. 2010).

Conclusion

The number of sporophytes in bryophytes is correlated with distance from geothermal features at Lassen Volcanic National Park, suggesting that sexual reproduction is reduced under extreme stress. These results support earlier work in fungi and mosses that showed a similar decrease in sexual reproduction in extreme-stress conditions (Huttunen 2003; Kis-Papo et al. 2003; Stark et al. 2005), despite a general trend toward an increase of sex with more moderate levels of stress (Iglesias and Bell 1989; Zeyl and Bell 1997; Greig et al. 1998; Grishkan et al. 2002). Future work in additional eukaryotes (e.g., algae and fungi from geothermal systems), would help determine whether the pattern holds, with sexual reproduction peaking at moderate to high levels of stress and then decreasing at the extreme edge of eukaryotic life. While this study analyzed rates of sexual reproduction using reproductive structures, additional data could be gained using molecular markers to determine genetic diversity across stress gradients with the assumption that increased sexual reproduction correlates with increased genetic diversity. Currently, genetic markers are limited for many of the geothermal bryophytes, but rapid development of microsatellite markers is making multiple-species, genetic structure studies a possibility in the near future.

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