Pollen quality for pollinators tracks pollen quality for plants in *Mimulus guttatus*

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Abstract. Pollen viability ranges substantially among and within plant species, and can be reduced by various factors, including pollution and inbreeding. While it is evident that a reduction in gamete quality reduces the male component of plant fitness, the implications of changes in pollen viability on the nutritional value of pollen for pollinators has not been studied. To test this, we created a greenhouse population of *Mimulus guttatus* plants that ranged in pollen viability from 17% to 98.5% and related both pollen quantity and pollen protein content to pollen viability. We found that crude protein concentration ranged from 15% to 45% and was positively associated with pollen viability. We also found that pollen mass per flower ranged 5-fold, also positively related to pollen viability. Across experimental plants, there was an 11-fold difference in protein mass per flower between plants of the lowest and highest pollen viabilities. We conclude that conditions that lead to reduced pollen viability, especially early in pollen development, may greatly reduce the awards available to plant pollinators.

Key words: inbreeding; pollen protein; pollen quality; pollination; pollinators; viability.

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INTRODUCTION

Wild pollinators play important and sometimes dominant roles in the production of insect pollinated crops, as well as provide key pollination services to wild plants and the food chains that depend on them (Ricketts et al. 2004, Kremen et al. 2007, Winfree et al. 2008, Julier and Roulston 2009). Recently, however, many concerns have arisen about how land management and other environmental impacts affect pollinator populations. Factors already identified include loss of habitat (Bates et al. 2011, Roulston and Goodell 2011), insecticide use (Brittain and Potts 2011), loss of preferred food plants (Biesmeijer et al. 2006) and the introduction of novel parasites (Cameron et al. 2011, Li et al. 2011). Little attention has thus far focused on the potential for environmental changes to alter the quality of host plants that pollinators use as food.

Pollen is the primary protein source for many pollinators, especially bees (Gilbert 1972, Howell 1974, Gilbert 1981, Erhardt and Baker 1990, Michener 2000, Roulston and Cane 2000). Larval bees of nearly all species acquire protein exclusively from pollen, and adult females require a protein diet to maintain egg production (Michener 2000). Pollen protein concentration ranges from 2.5% to 61.7% protein by dry weight, and increased protein has been associated with improved larval development in some pollinators (Roulston and Cane 2000, Roulston et al. 2000). Thus, ecological or environmental factors that influence pollen protein levels in plants have
the potential to influence the development of pollinators that rely on pollen as their protein source.

Current knowledge of variation in pollen protein concentrations is limited mainly to the range of variation across plant taxa, where it is evolutionarily conserved (Roulston et al. 2000). Little is known about the amount of variation within plant taxa or what factors may increase or decrease protein content. While it is unclear if there is any simple functional relationship between the amount of crude protein in pollen grains and any single role in plant reproduction (Roulston et al. 2000), substantial protein content comprises enzymes known to be involved in pollen tube growth and subsequent fertilization (Paton 1921), and these enzymes accumulate in the pollen grain during development (Gorenstein et al. 1991). Any disruptions to pollen development, therefore, could result in reduced enzyme production and lower protein content.

Pollen viability, the ability of pollen to germinate and grow, varies greatly among and within plants. Reduced viability is often associated with processes such as hybridization and inbreeding (Carr and Dudash 1997, Golmirzaie et al. 1998, Melser et al. 1999, Busch 2005, Glaettli and Goudet 2006, Epplley and Pannell 2009, Bures et al. 2010), or environmental stress such as exposure to high temperature or pollutants (Handique and Baruah 1995, Tretyakova et al. 1996, Gottardini et al. 2008, Pasqualini et al. 2011).

Pollen can become inviable either during initial pollen formation in the flower, resulting in malformed grains lacking some or all contents (Wilcock and Neiland 2002, Pipino et al. 2011), or after relatively complete pollen formation (Comtois and Schemenauer 1991). While these mechanisms yield roughly equivalent outcomes for the plant (failed fertilization), the timing of developmental failure could have important implications for the quality of pollen as animal food. As pollen grains develop, they acquire nitrogen transported from other tissues (Yuan et al. 2009). This nitrogen fuels a burst of DNA transcription and protein translation prior to anthesis that readies the pollen grain for the task of growing through stylar tissue and fertilizing ovules (Bedinger 1992, Lalanne et al. 1997). Developmental failure during this time would likely lead to pollen grains of lower overall mass, reduced nitrogen, reduced protein, and diminished nutritional value.

Here we pose two questions regarding pollinator rewards in *Mimulus guttatus* DC., a bee-pollinated plant species with varying levels of pollen inviability: (1) Is pollen viability related to protein concentration? (2) Is pollen viability related to pollen mass per flower?

**MATERIAL AND METHODS**

**Study organism and creation of breeding/pollination treatments**

We used the seep monkey flower, *Mimulus guttatus* (Phrymaceae) as our study organism. It is a bee-pollinated, annual/perennial herb native to wet areas across much of western North America (Kiang 1972). It produces minute quantities of nectar (Robertson et al. 1999), but substantial pollen, which is readily collected by bumble bees (Ivey and Carr 2005). Wild populations differ widely in mating system, with selfing rates ranging from 10% to 76% (Ritland and Ganders 1987). There are differences in pollen viability among populations in the wild, and forced inbreeding results in a steep decline in pollen viability (Carr and Dudash 1997). A loss of pollen viability occurs early in pollen development, resulting in small, shriveled pollen grains that fail to stain in aniline blue in lactophenol (Kelly et al. 2002).

The plant material used for this experiment originated from wild collected seed from one field population near Snell Valley, Napa County, California (38°42′06″ N, 122°24′29″ W). Thirty seeds from each of twenty maternal lineages were sown in the greenhouse, and one plant from each lineage was subjected to replicated hand pollination treatments (outcross, half-sibling cross, and self). Offspring from these crosses were grown to maturity and assessed for inclusion in analyses. There were two constraints on choosing test plants: because substantial pollen was required for protein analysis (1 mg per sample, 3 samples per analysis), plants had to produce many flowers (see *Crude protein content analysis* below), and their flowers had to produce a substantial amount of pollen. Some highly inbred lines produced insufficient pollen to collect, regardless of the number of flowers.
Overall, 40 plants were used for analysis.

Pollen viability assay

Two to four flowers per plant were harvested and two anthers from each flower were removed and submerged in 0.1% aniline blue in lactophenol. Darkly stained grains were considered viable; unstained or very lightly stained grains were considered inviable. Variability among flowers on a single plant was low and the mean staining value was used to represent a plant’s pollen viability. Pollen stainability indicates well developed cytoplasmic and nuclear material and has been used commonly as an index of pollen viability across plant species in general (Hauser and Morrison 1964) and *Mimulus* specifically (Carr and Dudash 1997, Willis 1999, Kelly et al. 2002, Vallejo-Marín 2012, Fishman et al. 2013). Although we didn’t compare methods for determining viability in *Mimulus guttatus*, the results from this technique generally are highly correlated with the use of tetrazolium blue, which stains for respiratory activity of pollen grains (Hauser and Morrison 1964).

Crude protein content analysis

To determine the concentration of pollen protein, the Bradford Protein Assay was used (Bradford 1976). All flowers (mean: 86.4, range: 30–276) from each test plant were removed and sampled. The cut flowers were allowed to sit overnight in the greenhouse allowing the pollen to dissociate from the anthers, which yielded more pollen than fresh flowers. Pollen was collected using a modified electric toothbrush to shake the pollen free from the anthers onto a plate of glass. Pollen was then fully dried overnight in a drying oven at 40°C and stored in a freezer until analyzed. Pollen samples from the same plants were combined after drying to make a single bulk sample per plant. Following Roulston et al. (2000), 1-mg samples of dried pollen (3 replicates per estimate) were ground with aluminum powder in a mortar and pestle to break the exine and release the cytoplasmic contents. Pollen protein was extracted overnight in 0.1N NaOH. The following day, Commassie Blue dye, which binds to protein, and polyvinylpyrrolidone, which absorbs phenols that might interfere with protein binding, were added to the samples. The absorbance of the sample solutions was measured on a spectrophotometer at 595 nm and compared to standards of known protein concentration. The protein standards were made by using different amounts of *Typha latifolia* (cattail) pollen prepared like the sample pollen. The protein concentration of *Mimulus guttatus* pollen was calculated using linear regression to interpolate protein values based on absorbance values of the standards. An additional pollen (*Helianthus annuus*) of known protein content was run with each set of samples to assure accuracy of the standard line.

Statistical analysis

All statistical analyses were run in R (R Development Core Team 2012) using package nlme for linear and non linear mixed effects models. Choosing only the individual plants that were productive enough to use in protein analysis created imbalance in the dataset regarding the maternal family of the chosen plant and the pollination treatment of its maternal donor. Thus, it was not possible to run a full factorial model that included maternal family, pollination treatment, and viability as predictors. Instead, we used pollen protein as the dependent variable, the maternal family × maternal pollination treatment combination as a random factor, and pollen viability as a covariate. The dataset for comparing pollen production per flower (dry weight of all pollen collected per plant/total flowers harvested per plant) with pollen viability is greatly reduced (only 10 plants) because many leftover pollen samples (following protein analysis) that were insufficient for additional analyses were inadvertently discarded before being weighed. The fully quantified samples came from one maternal family, three with replicates, and we analyzed the relationship between pollen production per flower and pollen viability by using pollen production per flower as the dependent variable, maternal family × maternal pollination treatment as a random factor, and pollen viability as a covariate. Protein production per flower was analyzed using a statistical model similar to pollen production per flower. Although we were not able to test the effect of plant family and breeding treatment on protein concentration and plant productivity due to the strong imbalance in the subset of plants analyzed, we did find a significant effect of both
plant family and breeding treatment on pollen viability in the overall set of plants from which the subset was chosen (T. H. Roulston, unpublished data), similar to other studies on *Mimulus guttatus* (e.g., Carr and Dudash 1997). Our statistical approach accounted for this relationship between inbreeding and pollen viability (through the inclusion of the maternal random effect), but the approach did not directly test for an inbreeding effect on viability.

**RESULTS**

Pollen was hand collected from a total of 3456 flowers across the 40 treatment plants. Crude pollen protein ranged from 15% to 45% among individual plants and increased with pollen viability ($F_{1,16} = 13.48, P = 0.002$; Fig. 1). Pollen viability showed a statistically significant positive relationship ($F_{1,5} = 7.28, P = 0.042$) with pollen productivity, ranging from 0.01 to 0.05 mg pollen per flower (Fig. 2). Pollen protein concentration multiplied by the amount of pollen produced per flower provides an estimate of protein per flower. Protein per flower increased with pollen viability ($F_{1,5} = 8.41, P = 0.034$), with the most productive plant containing over 11 times as much protein as the least productive plant (Fig. 3).

**DISCUSSION**

*Mimulus guttatus* plants of higher pollen viability have both increased concentrations of pollen protein and increased amounts of pollen overall. Bees foraging on the least productive,
lowest viability plants would have to visit over eleven times as many flowers as bees foraging on the most productive, highest viability plants in our study to collect an equal amount of protein (Figs. 1 and 2). Thus, for *Mimulus guttatus*, and likely other plant species in which conditions can bring about developmental failure in substantial amounts of pollen, conditions that reduce pollen viability may substantially reduce the value of plants for their pollinators. One environmental condition that is both increasingly common and relevant is habitat fragmentation; many studies have shown that habitat fragmentation reduces plant population sizes and increases inbreeding (Ellstrand and Elam 1993, Schleuning et al. 2009), and inbreeding is commonly associated with reduced pollen viability (Carr and Dudash 1997, Golmirzaie et al. 1998, Melser et al. 1999, Murren 2002). It remains to be seen whether the relationship described here also occurs when pollen viability is reduced after development is mostly complete, such as when pollen is exposed to pollution after anthesis (Pasqualini et al. 2011, Duro et al. 2013).

How pollen collecting pollinators respond to low quality pollen when foraging has been examined in only a few studies. Robertson et al. (1999) found that in a greenhouse experiment bumble bees chose to forage on *Mimulus guttatus* plants with higher viability pollen, but they did not consistently discriminate among plants in the field, presumably because competition for resources among bees led them to visit all flowers of preferred hosts. Ivey and Carr (2005), also working with *Mimulus guttatus*, for which a relationship between inbreeding and viability has been previously established (Carr and Dudash 1997), found that inbred plants received fewer visits by pollinators, but they did not examine pollen viability in that study. Bumble bees increased foraging by 55% and pollen storage by 233% when they were presented with plants of higher quality pollen (pure pollen) instead of low quality pollen (pollen diluted with cellulose) (Kitaoka and Nieh 2009). In research looking at bee preferences across different plant species, it was found that bumble bees chose to collect pollen from plants with greater concentrations of protein in their pollen (Hanley et al. 2008), even after controlling for plant phylogeny. In a study that combined pollen protein concentration and average standing crop of pollen, it was found that bumble bee foraging preferences were related to standing crop of protein (Rasheed and Harder 1997).

If pollinators can recognize inferior resources and avoid plants that present them, then they may exert selection pressure against plants of low pollen viability when reduced viability is genetic in origin. Plants with substantial amounts of inviable pollen already pay a cost to the male component of fitness by siring fewer offspring on other plants and potentially siring fewer seeds through self pollination (at least in self-compatible lineages). The addition of pollinator discrimination would compound existing selection against genotypes that produce low quality pollen. Sudden loss of a pollen vector can lead to the rapid evolution of a selfing phenotype in self-compatible species (Roels and Kelly 2011), but it may not lead to a quick reduction in the alleles causing low pollen fertility. In *Mimulus guttatus*, reductions in male fertility are caused substantially by numerous alleles of mild to moderate effect, which are not quickly purged (Carr and Dudash 1997, Willis 1999). If pollinators avoid plants of low pollen viability, they will acquire high quality resources for themselves and select for plant genotypes that provide it.

This is the first study that shows the extent to which crude protein concentration can vary within plant species. Protein in pollen is known to range from 2.1% to 61% dry weight across species (Roulston et al. 2000). For bee-pollinated plant species, pollen protein generally ranges from 12% protein to 61% protein, with great uniformity within species and within closely related taxa (Roulston et al. 2000). In this study of one species, pollen protein ranged from 15% in the lower viability plants to 45% in the higher viability plants. Even the lower viability plants analyzed here are within the normal range of pollen protein levels for bee pollinated plants, and therefore are still potentially useful for bees. Plants of the lowest viability levels that resulted from experimental crosses, however, could not be analyzed due to insufficient pollen and it is probable that both nutritional level and resource abundance are inadequate at the lowest viability levels that can occur in *Mimulus guttatus*.

Although our experimental design didn’t allow for a sensitive test of the effect of inbreeding on
pollen viability, the relationship is already well known for *Mimulus guttatus* (Carr and Dudash 1997). We chose plants primarily to maximize the range of pollen viabilities tested, limited to those plants that produced sufficient flowers and pollen for analysis. Given that the relationship between protein concentration and viability was detectable taking a conservative approach of reducing the data set to genotype × pollination groups, we conclude that our result is quite robust: low quality pollen, as evidenced by viability and protein, is low quality pollen regardless of the route that it took to get there.

This study demonstrates how ecological factors that modify pollen viability have the potential to reduce the quality of food for pollinators. Future work should focus both on the circumstances under which these ecological factors do alter food quality and how the interactions between pollinators and their altered hosts affect pollinator populations, plant populations and selection on plant traits.

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