Measuring the agents of natural selection is important because it allows us to understand not only which traits are expected to evolve but also why they will evolve. Natural selection by pollinators on floral traits is often assumed because in outcrossing animal-pollinated plants flowers are generally thought to function as advertisements of rewards directed at pollinators. We tested the role of bee pollinators in selection on *Penstemon digitalis* and found that pollinators were driving selection for larger and more flowers. However, what makes our publication unique is the additional information we gained from reviewing the few other studies that also directly tested whether pollinators were agents of selection on floral traits. As we would expect if pollinators are important agents of selection, selection on floral traits was significantly stronger when pollinators were present than when their choices were experimentally removed. Taken together, these results suggest that pollinators can be important drivers of selection in contemporary populations.

Plants and Pollinators

In a recent publication, we show that pollinators can drive selection on floral traits of *Penstemon digitalis*. Moreover, we found that although there are few studies available that directly test whether pollinators are the agents of natural selection, selection on floral traits is generally stronger in the presence of pollinators than in their absence. These results are important because they both empirically demonstrate that pollinators can and do act as agents of selection in the wild and that pollinator-mediated selection in contemporary populations may be stronger than generally suggested.

For many plants, pollinators are crucial for their sexual reproduction. Thus, pollination biologists have been long interested in the interactions between plants and their pollinators. It is often assumed that these interactions result in pollinator-mediated natural selection on floral traits. However, a major gap in our understanding of floral evolution, especially these micro-evolutionary processes, is the exact role of pollinators in generating patterns of natural selection on floral traits. Although pollinators seem to be important drivers of floral evolution on a macro-evolutionary scale and natural selection on floral traits is common (although not consistent) on a micro-evolutionary scale, surprisingly few studies have tested whether pollinators are the agents of natural selection within populations.

It is only through experimental manipulation of an agent that one can determine whether or not they are driving selection on a particular trait. Generally, to test whether an agent is driving selection on a trait the most definitive approach is comparing natural selection in the presence and absence of that agent, ideally by experimentally removing the agent. In the case of pollinators, excluding pollinators does remove their influence on fitness but because they are directly involved with reproduction this will cause complete reproductive failure for the plant or only self-pollination. Because we are interested in what natural selection would be when all plants receive ample pollen for reproduction rather than pollen delivery based on pollinator preferences, hand-pollinations act to remove pollinator influence on plant reproduction and offer a base-line...
population to compare to open-pollinated plants. If natural selection is significantly stronger in open-pollinated rather than hand-pollinated plants, then we interpret this difference as the pollinator-mediated selection on that trait.

*Penstemon digitalis* offered an ideal system to study pollinator-mediated selection because pollinators have been strongly implicated in diversification of the genus,7 however, natural selection has not been measured for any of the species. Therefore we experimentally tested whether pollinators were an agent of selection on floral traits in this species.1 To see whether we could generalize our results to flowering plants, we reviewed studies that measured pollinator-mediated selection in other plant species. In our publication, we found six studies that measured natural selection in open and hand-pollinated plants.8-13 Here we included an additional study which was recently published alongside our paper14 as well as data from an upcoming publication15 and one that was brought to our attention.16 With the additional selection coefficients we can perform a more informed test of whether selection on floral characters is generally stronger in open-pollinated plants when compared to hand-pollinated plants. We also included selection studies that manipulated herbivores and co-flowering species and measured selection on floral characters to compare these agents with pollinators.1

**Pollinator-mediated Selection**

We found significant pollinator-mediated selection for larger flower size and greater flower number in *P. digitalis*.1 Flower size and number are often under positive natural selection1 and pollinator behavior suggests that they often prefer to visit plants with large showy displays because these plants have greater rewards.17 These two components, pollinator preference for large displays and natural selection for larger displays, has suggested that pollinators must be the agents of selection on these traits. However, surprisingly few studies have shown that pollinators are the agents of selection12,14 suggesting that pollinator preferences do not always translate into fitness differences for the plants. Furthermore, other studies have found significant selection on flower size and number but have experimentally demonstrated that pollinators are not driving this selection.9,13 Thus, it is clearly not enough to measure selection on a trait and assume the agent of selection based on behavioral experiments.

Although many of the reported selection coefficients in open and hand-pollinated treatments were close to zero and non-significant, we found that overall selection on floral traits was stronger in the presence of pollinator choice rather than its absence (Fig. 1). Additionally, our updated analyses showed an even stronger tendency for selection to be stronger in the presence of pollinators than our previously published result ($F_{1,39} = 7.29, p = 0.01$ vs. $F_{1,22} = 5.78, p = 0.026$). Moreover, when we compared pollinators to co-flowering plants (competitors or facilitators) and herbivores, we found that pollinators were a stronger agent of selection on floral traits.1 A recent and comprehensive review of selection on floral traits indicates that phenotypic selection on floral characters is often sporadic, suggesting that pollinators are unlikely to be strong selective agents.3 However, our review suggests that even with many non-significant selection coefficients, the general pattern is strong.

**Accounting for Plant Vigor in Selection Analyses**

Correlations among traits may mask direct selection on any given trait.18 Thus measuring selection using multivariate statistics has greatly enhanced our ability to detect direct selection on phenotypic traits and therefore the targets of selection. However, a frequently ignored (or at least not often discussed) aspect of natural selection studies is that the outcome of the analyses and therefore our conclusions can depend on which traits are included in our model. For example, when we included only traits which we hypothesized pollinators could be directly driving selection on (flower size, petal color, total number of flowers, flower density, aborted flowers and inflorescence height) in our analyses, we found no significant differences in selection on flower size or flower number between open and hand-pollinated *P. digitalis*. This would have led us to conclude that pollinators were not agents of selection on these traits. However, the addition of a plant vigor trait (above ground biomass) in the model gave us the power to detect differences between our treatments and led us to conclude that pollinators were the agents of selection on flower size and number.1 It is important to note that the general trend was similar in both analyses (selection was stronger in open-pollinated plants), however, as evolutionary ecologists we generally base our conclusions on whether a difference is statistically significant or not therefore the difference between the analyses would lead to contrasting conclusions.
Generally we expect vigor to be a strong predictor of fitness and positive selection on plant vigor traits is common. However, we often do not directly consider plant vigor when studying selection by pollinators (reviewed in ref. 10). Natural selection is generally weak in wild populations and although this can still lead to significant evolutionary change, in any particular population we expect there to be subtle relationships between traits and fitness. Furthermore, we generally expect plant species to be adapted to their pollination environment. Thus an approach that increases the likelihood of detecting selection in wild populations would be advantageous.

The benefit of including traits related to vigor in natural selection studies focused on other aspects of the phenotype is two-fold. First, if there are correlations between vigor and other traits, including vigor in the model will give a better estimate of direct selection on the traits the study is focused on. Second, even if there are no correlations among the traits of interest and vigor, the addition of a strong predictor of fitness to the model will help explain variation that would otherwise be included in the error term. A smaller error will increase the power to detect selection on the traits of interest. Thus, accounting for strong predictors of fitness such as plant size may allow researchers to detect patterns of selection that would not be apparent otherwise and may reveal additional insights into underlying selection mechanisms. Some of the studies we reviewed accounted for plant vigor by measuring a plant size component such as height, leaf weight or rosette area, while others did not. Thus the inclusion of traits representing plant vigor in the analyses may influence the interpretation of some of the results in the reviewed studies. Regardless, the differences in natural selection coefficients that we found when we accounted for plant vigor or not suggest that studies of selection on floral traits should include plant vigor in their models.

**Conclusions**

The general assumption that pollinators are major drivers of selection on floral traits have been confirmed by our results. However comforting, these data are limited (especially to bee and fly pollinated herbaceous plants), suggesting that there is still much work to be done to further our understanding of floral evolution. Of particular interest is examining selection by vertebrate agents, selection in woody species and tackling selection by pollinators through male fitness in hermaphrodite plants. As Harder and Johnson suggest, selection by pollinators may be easier to detect in environments where some significant change is occurring (such as introduced species, disturbance to the pollinator network, etc.). We also strongly encourage including a measurement of plant vigor (appropriate for the system) when measuring selection in any system or by any agent. A combination of these approaches may help untangle the role of pollinators in driving current selection on floral characters.

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