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Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub

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Abstract

The coexistence of different color morphs is often attributed to variable selection pressures across space, time, morph frequencies or selection agents, but the routes by which each morph is favored are rarely identified. In this study we untangle the interactions and trait pleiotropisms that influence floral color polymorphisms on a local scale in *Protea*, within which ~40% of species are polymorphic. Previous work shows that seed predators and reproductive differences likely influence polymorphism maintenance in four *Protea* species. Here, we explore whether selection acts on floral color directly or indirectly in *Protea aurea*, using path analysis of pollinator behavior, nectar production, seed predation, color, morphology, and fitness measures. We found that avian pollinators spent more time on white morphs, likely due to nectar differences, but this had no apparent consequences for fecundity. Instead, a single continuous trait underpinned many of the reproductively-important differences between color morphs: the number of flowers per flowerhead. This trait differed between color morphs (white had more), and it was also positively correlated with nectar, seed predation, and fecundity independent of predation. Realized fecundity, in contrast, was not directly associated with color or any other floral trait, although it covaried with leaf chlorophyll content, which was higher in white morphs. Thus, although conflicting selection via predation and higher reproductive potential may promote color polymorphism in *Protea aurea*, the phenotypes targeted by selection are more strongly associated with the number of flowers per head, leaf chlorophyll content, and their unmeasured correlates, rather than with flowerhead color itself.

Keywords: anthocyanin; correlational selection; polymorphism; path analysis; Protea aurea

Abbreviations. SLA: specific leaf area (cm^2 g^{-1})

Introduction

Phenotypic polymorphisms are visually striking examples of the genetic variation that underlies evolutionary change, and as such, their maintenance has long been of research interest (e.g., Wright 1943; Haldane 1949; Levene 1953; Levins 1969; Hedrick 1986; Hedrick 2006). The evolutionary processes that favor polymorphism are diverse, ranging from selection pressures that vary over space, time, selection agents, or with morph frequency–i.e., frequency dependent selection–, to heterozygote advantage. Polymorphisms may also persist without selection if populations are large and mutation between morphs is frequent (Falconer 1989). Among these processes, the most commonly implicated are negative frequency dependence (e.g., Greenwood 1985; Barrett 1988; Gigord et al. 2001; Hiscock and McInnis 2003) and spatially variable selection regimes (Warren and Mackenzie 2001; Galeotti et al. 2003; Whitney and Stanton 2004; Schemske and Bierzychudek 2007). Variable selection *within* populations may also shape many polymorphisms, such as when the favored phenotype varies within or among classes of selection agents, often in association with pleiotropic effects (Jones and Reithel 2001; Strauss and Whittall 2006; Gray and McKinnon 2007; Rausher 2008).

Flower color polymorphisms provide particularly striking examples of how polymorphism can be maintained variable selection. The coexistence of multiple flower color morphs has been linked to variability in selection mediated by pollinators, antagonists or environmental gradients (Strauss and Irwin 2004; Strauss and Whittall 2006; Schemske and Bierzychudek 2007; Caruso et al. 2010). Pollinators are often implicated because their color preferences may differ among individuals, guilds, over time, or with morph frequency, thereby balancing the fitness of each color morph (Levin and Watkins 1984; Gegear and Laverty 2001; Gigord et al. 2001; Jones and Reithel 2001; Eckhart et al. 2006). Like pollinators, herbivores may discriminate among color morphs (Frey 2004; Johnson et al. 2008), and their feeding preferences may vary among sites, over time, or across taxa (e.g., Irwin et al. 2003; Strauss et al. 2004; Whitney and Stanton 2004). Together, the effects of pollinators and predators may ultimately conflict to promote polymorphism within populations, because the plants most visited by pollinators are likely to produce more food for pre-dispersal seed predators (Irwin and Strauss 2004; but see Caruso et al. 2010). Finally, variable selection along abiotic gradients is regularly implicated in the maintenance of flower color polymorphisms, in part because anthocyanins are linked to increased tolerance of extreme temperatures, pests, soil infertility, or UV radiation, but their production may also have costs (Koes et al. 1994; Warren and Mackenzie 2001; Steyn et al. 2002). For example, long-term surveys of *Linanthus parraye* demonstrate that fluctuating selection associated with rainfall is contributing to maintenance of a blue-white flower color polymorphism (Epling et al. 1960; Schemske and Bierzychudek 2001; Schemske and Bierzychudek 2007).

As most of the above examples suggest, the targets of the diverse selection pressures affecting polymorphisms are often not the presence or absence of pigmentation *per se*, but rather traits associated with pigmentation, either via genetic linkage or pleiotropy. Rigorous work on *Ipomoea purpurea*, for example, links white morph rarity to the detrimental pleiotropic effects of a mutation blocking anthocyanin production (Coberly and Rausher 2003; Coberly and Rausher 2008). Significant correlations between pigmented flowers and other fitness-related traits have been also been documented in other species (e.g., *Hydrophyllum appendiculatum*, Wolfe 1993; *Phlox drummondii*, Levin and Brack 1995; *Lobularia maritima*, Gomez 2000), and selection acting through those traits is broadly known as indirect, or correlational selection (Gomez 2000; Grey and McKinnon 2007). Together, these studies illustrate the wide range of selection pressures and pleiotropic effects that likely contribute to any given polymorphism, yet very rarely are these factors examined in concert or even in the same species (but see Frey 2004; Caruso et al. 2010; reviewed in Rausher

2008). The underlying mechanisms of variable selection and the actual targets of selection for pollinators and antagonists are unknown for the vast majority of polymorphic plant species.

Our previous research on four species of *Protea* in South Africa strongly suggests that spatially heterogeneous selection associated with seed predators helps maintain the pink to white inflorescence color polymorphism that is present in 40% of species in the genus (Carlson and Holsinger 2010). First, pre-dispersal seed predators ate more seed of white than pink morphs within half of study populations, and in the other half, predation was equal between morphs. Seed predators eliminated over half of potential seed set in some sites, but their effects varied with elevation and/or its environmental correlates. Second, pink morphs had more strongly pigmented stems than white morphs, indicating that differences in pigmentation extended throughout the plant. Third, seed mass was slightly but significantly higher in white than in pink morphs across the four species, and seeds of white morphs were also 3.5 times more likely to germinate. This association probably reflected a trait pleiotropism, but it could also have been associated with the costs of anthocyanin production (Steyn et al. 2002). Finally, seed set in the absence of predation was equivalent between color morphs, suggesting pollinators did not discriminate; however there exists no observational data on pollinator responses to different *Protea* color morphs, nor are there data on how nectar production influences bird preferences.

In the current study, we build on our previous work by untangling the web of interactions and trait pleiotropisms that influence floral color polymorphisms on a local scale. We explore whether polymorphisms are maintained through direct selection or indirect selection on correlated traits by taking detailed measurements of pollinator behavior, nectar production, and seed predation in two conspecific *Protea* populations. We then relate these data to floral color, other plant traits, and ultimately, plant reproductive success, to determine how selection pressures might interact to contribute to polymorphism maintenance. The local scale of the current investigation complements the broad spatial scale of our previous work and aims to identify mechanisms that could account for genus-wide patterns. Our focal research questions are:

1. Do pink and white morphs differ in nectar production rates, pollinator preferences, or seed predation? Does nectar production, bird behavior, or predation intensity vary between sites or over a flowerhead's lifetime?

2. If color morphs differ in biotic interactions or nectar production, are these differences directly associated with color, or are they mediated by correlations with other floral or vegetative traits?

3. Is floral pigmentation under balancing selection that favors each morph under different circumstances? If so, is color directly associated with potential or realized fecundity, or are color and fecundity indirectly related via biotic interactions or correlations with other traits?

Methods

Study species

We examined the links between flowerhead color, nectar, pollinators, predators and correlated traits in two populations of *Protea aurea* (Burm.f.) Rourke subsp. *aurea* in the Western Cape of South Africa. *Protea aurea* is an upright (up to 4 m), evergreen shrub endemic to the Langeberg, Outeniqua, and Riversonderend mountain ranges (Rebelo 2001). We studied *P. aurea* in two sites ~70 km apart in the Langeberg mountains, Marloth and Garcia's Pass. The elevations of the Marloth and Garcia's Pass sites are, respectively, 239 and 491 m a.s.l. and they receive 882 and 491 mm rainfall annually. While we were on-site, Marloth received 22.8 mm rainfall (mid-April to mid-May 2010) and Garcia's Pass received 8.4 mm (April 2011; data from adjacent towns; South African Weather Service 2011).

As for all other *Protea* species, habit and reproduction in *P. aurea* are associated with the semi-frequent fires that occur throughout the region (15-40 year intervals; Forsyth and van Wilgen 2007). Fire kills *P. aurea* adults, and their seeds are released from serotinous infructescences (henceforth seedheads) to germinate during the next rainy season. In both of our study sites, stand age was relatively young; Marloth burned in Dec. 2002 and Garcia's Pass in Jan. 2005. Although aboveground seed storage in *Protea* has the potential to produce large seed banks over time, seed predators often consume over 60% seeds on the plant (Carlson and Holsinger 2010). Common pre-dispersal seed predators of *Protea* include the stem-boring larvae of moths (*Synanthedon*, Sesiidae; *Tinea*, Tineidae; *Argyroploce*, Olethreutidae) and beetles (*Genuchus hottentottus*, Scarabidae; *Sphenoptera*, Buprestidae; *Euredes*, Curculionidae; Coetzee and Giliomee 1987a; Coetzee and Giliomee 1987b; Wright and Samways 1999; Roets et al. 2006). Even when seed predators are absent, however, the genus as a whole is characterized by low seed to ovule ratios: in *P. aurea*, only 11% of ovules develop into viable seeds (n=104 plants in three sites; Carlson and Holsinger 2010).

The floral biology of *P. aurea* closely resembles that of most *Protea* species. Individual flowers consist of a solitary ovule, a reduced perianth that is fused with the anthers, and a single, long style that also serves as a pollen presenter. Nectar is produced by two small nectaries located within the perianth at the base of each flower. Flowers are grouped onto broad, cup-like inflorescences attached to woody receptacles, i.e., flowerheads or heads, and subtended by large, petal-like bracts that range in color from deep pink to pure white. In most species of *Protea*, flowers are protandrous and development is staggered such that both sexual phases often co-occur within flowerheads (Collins and Rebelo 1987). In *P. aurea*, however, all flowers in a head appear to mature and dehisce pollen simultaneously (J. Carlson, pers. obs.), which may be possible because flowerheads contain relatively few flowers (70-80). Inflorescence development begins on Day 1, when the bracts spread open, but pollen presenters are not yet exposed. On Day 2, all pollen is dehisced as the perianth tube snaps away from the pollen presenter, often as a pollinator lands in the flowerhead. Maximal stigma receptivity occurs at least 1-3 days after the perianth has fallen away, as evidenced by hand pollination experiments and changes in stigmatic features of various *Protea* species (Vogts 1971; Van der Walt and Littlejohn 1996; Littlejohn et al. 2001). Our observations of *P. aurea* suggest that its female phase spans Days 3-4, after which styles begin to shrivel and turn brown, suggesting they are no longer receptive.

Protea aurea blooms between January and June, although a few flowerheads may be present at most times of year (Rebelo 2001). In our study populations, peak flowering was observed between April and June. In our Garcia's Pass site, flowering overlapped with three co-occurring bird-pollinated *Protea* species, two of which are also color polymorphic, *P. neriifolia* and *P. repens*, and one species, *P. eximia*, that is not color polymorphic. In our Marloth site, in contrast, *P. aurea* was the only *Protea* species observed in full flower. Both populations were dominated by individuals with white-flowerheads. Based on visual surveys over the course of the study, we estimated pink-morph frequency to be 5-10% at Marloth and 25-30% at Garcia's Pass.

Sampling design

Nectar measurements—We measured accumulated nectar volumes and sugar concentrations in the flowerheads of 14-19 pink morphs and the same number of white morphs in each site (n=64 plants total). To start we bagged two or more unopened inflorescences (flowerheads) per plant, and as they opened, we randomly assigned them to be measured on the morning of Day 2 or Day 4. On the assigned morning, we picked the flowerhead and immediately measured its total length, diameter at base, and counted the number of flowers per head. We then measured nectar by repeatedly filling a 20 μL microcapillary pipette inserted into the base of each flower until all nectar was extracted from all flowers. We used a refractometer to measure sugar concentration (w/w) after the full volume was extracted and re-combined. Although the nectars of *P. aurea* and other bird-pollinated *Protea* are known to contain more glucose and fructose than sucrose (Cowling and Mitchell 1981; Nicolson and Van Wyk 1998), we were unable measure the relative amounts of mono- and di-saccharide sugars in the field and provide only the concentration of all three sugars combined (Kearns and Inouye 1993). We calculated the mass of sugars in the nectar by first converting sugar concentration to grams of sugar per liter of nectar (Kearns and Inouye 1993) and multiplying that by nectar volume. We excluded bagged flowerheads that were open during a rainfall event or from which nectar had leaked or was spilled, which resulted in a disproportionate loss of Day-4 heads (Day 2=66; Day 4=27).

We also measured nectar replenishment rates of individual flowers on seven plants per color morph in the Marloth study site only. All of the nectar replenishment plants were also in the nectar accumulation study, although each study used a different set of bagged flowerheads. We focused on replenishment from 10 AM to 4 PM because peak production occurred during those hours (J. Carlson, unpublished). To measure replenishment, we selected and marked two flowers near the center of a newly open flowerhead, and we sampled them at 10 AM and 4 PM each day for four consecutive days. The first time a flower was sampled, we cut a small slit in the perianth base and gently inserted a 10 μL microcapillary tube to remove all nectar, after which we rebagged the flowerhead. We measured the volume and sugar concentration as above. For each subsequent flower sampling, we used the same slit to minimize perianth damage. We calculated the mean hourly nectar replenishment rate during day-time hours (10-4) by taking the volume measured at 4 PM, averaged across the two flowers per flowerhead, and dividing it by the six hour replenishment interval. At the end of the 4-day period, we counted flowers per flowerhead and measured total flowerhead length and diameter at base.

Pollinator preferences—We video-recorded avian pollinator visits to individual plants and flowerheads of *P. aurea* for 18 days at Marloth and 20 days at Garcia's Pass. Each day, we chose a pair of similarly-sized plants with near-equivalent floral displays that were growing close together, one with pink flowerheads and the other with white flowerheads. We avoided plants that were in current use or had been used for nectar measurements within the last week so as not to disturb pollinators. As a result, we were only able to collect both nectar and video data from 23 plants, all at Marloth, and 11 of our study plants were recorded twice (65 plants total). For each recorded plant, we counted all flowerheads and estimated the age of each flowerhead as Day 1: Pre-male phase, Day 2: Male phase, or Days 3-4: Female phase. We targeted plants with at least two or three open heads, preferably one of each phase, but were often limited by what was simultaneously available on a pink and white morph each. At Marloth, individual displays were larger on average, allowing us to recorded 364 heads total: 84 in Day 1, 57 heads in Day 2, and 223 in Days 3-4. In Garcia's Pass, the total was lower at 180 (31, 66, and 83 respectively). The two color morphs were recorded simultaneously with a separate camera for 3-4 h per day, split into two sessions starting at ~9 AM and 2 PM. While reviewing video data, we identified each avian pollinator that approached the plant, and we recorded the number of flowerheads visited (*i.e.*, actively fed from) and length of time spent feeding each flowerhead prior to its departure from the plant. Because we were unable to distinguish most female sunbirds in the video, we grouped all sunbird visitors together, but otherwise we recorded species identity whenever possible. Although bees were also seen visiting flowerheads, we did not quantify their visits because they were difficult to track and they are unlikely to be effective pollinators of *P. aurea* (J. Carlson, pers. obs.).

Seed predation—We examined rates of infestation and damage in 92 *P. aurea* color morphs from both of our study sites. Our sampling included most of the plants sampled for nectar (49), half of those sampled for video (32), and 24 additional plants. From each plant, we collected 3-5 seedheads that were 2-3 years old. After seedheads dried to opening, we counted the number of seed that were undamaged and contained endosperm. All heads were then were coded as with or without evidence of infestation, i.e., presence of a larva, frass, or damage to the seedhead base or interior. We also counted the number of flower attachment points on a photograph of the dried receptacle, which corresponds to the number of flowers counted on fresh heads. Occasionally, receptacles were too damaged for flower counts (20% of all heads), but most had little to no damage (60%) or had enough of the base intact for reliable estimates (20%); infested heads with some versus no visible damage had similar mean flowers per head, confirming count reliability (J. Carlson, unpublished). Finally, we calculated the percent of heads infested as the number of seedheads that showed evidence of infestation divided by the total number of seedheads examined. The degree of damage was estimated as the number of seeds present in undamaged minus damaged seedheads.

Whole-plant measurements—We collected morphological and performance-related data on all 92 of the plants measured for seed predation, thereby including many of the nectar and/or video plants. On each plant, we counted growth intervals as an estimate of plant age, measured total plant height, and counted the number of seedheads and flowerheads as an estimate of current and past investment in reproduction and pollinator attraction (henceforth total heads per plant). We also weighed all undamaged seeds from the dried seedheads collected for seed predation measures, and calculated for each plant the mean mass of a single seed. Finally, we estimated both potential and realized maternal fecundity by multiplying the number of heads per plant by the number of seed per head. Potential fecundity was based on seed counts only in uninfested heads, whereas realized fecundity was based on all examined heads. These values serve as an estimate of the total number of seeds produced over the plant's lifetime thus far, i.e., long-term fecundity. On a subset of nectar plants at Garcia's Pass only, we collected from each plant a single, fully expanded leaf from last-year's growing season. On this leaf, we measured specific leaf area (leaf area divided by leaf mass in cm^2g^{-1}; SLA) and an index of leaf chlorophyll content with the hand-held CCM-200 plus (Opti-Sciences), averaging two CCM measurements per leaf.

Statistical analyses

Q1: Are there differences in nectar, pollinators, and seed predators between colors, sites, and over time?—We compared rates of nectar accumulation and replenishment in flowerheads of pink and white morphs in linear mixed models with color, day of measurement, and their interaction as fixed effects. For nectar accumulation, we also included the fixed effects of site and its interactions. Random effects were source plant, sampling date and estimated age, which controlled for ontogenetic differences. We used a similar model to compare nectar replenishment rates between 10 and 4 PM at Marloth, except that we made adjustments to account for repeated measures on the same flowers over time (repeated statement in Proc MIXED, type=ar(1)). We performed two pre-planned contrasts to compare nectar replenishment in male-phase heads (Day 2) to that of each age class of female-phase heads (Days 3 and 4). For the above replenishment and accumulation analyses, we compared rates of nectar production in terms of nectar volumes (µL nectar), mass of sugars (mg sugars), and sugar concentration (w/w).

We ran two additional nectar analyses to determine first, whether nectar accumulation or refill rates were also associated with traits of the measured flowerheads, and second, whether the two nectar measures were correlated. For the first analysis, we used linear mixed models with the same fixed and random effects as above, with the added covariates of the individual flowerhead's length and flowers per head. We did not include flowerhead base diameter in any analyses because it was tightly correlated with flowers per head (Pearson's R=0.67 for 117 flowerheads). For the second analysis, we tested the correlation between nectar accumulation (predictor) and nectar replenishment (response) in a MIXED model that accounted for repeated measures on flowerheads within plants, and also included the random effects of day of measurement, the number of flowers per head, color, and color × day.

We compared avian pollinator behavior on pink and white morphs using repeated-measures mixed and generalized linear mixed models on pollinator visit rate and visit duration. We performed separate analyses on data collected at the per-plant and per-flowerhead levels. In the per-plant analysis, visit rate was the was number of times a bird arrived to a plant and visited at least one head prior to departure, and visit duration was the number of seconds spent feeding from a head, averaged across all heads visited during the recording session. In the per-flowerhead analysis, visit rate was the number of visits to an individual flowerhead, and visit duration was the mean time spent feeding from that flowerhead. Fixed effects for per-plant analyses were site, morph color, time of day (AM or PM), and interactions. Fixed effects in the per-flowerhead analyses were site, color, flowerhead age (1, 2 or 3-4 days old), and interactions. Time of day was a random effect in per-flowerhead analyses because visit rates to individual heads were too low for within-day comparisons. In both per-plant and per-flowerhead analyses of color preferences, visit rate was modeled using a Poisson distribution. Visit duration required a square-root transformation to normalize residuals in the per-flowerhead analysis. In all models, we included random effects accounting for repeated measures on the same plant or flowerhead in the morning and afternoon (SAS 9.3; GLIMMIX manual, Littell et al. 2006; SAS Institute Inc. 2008). We also included in both models the random effects of sampling date, the number of flowerheads per plant, and interactions between sampling date and color.

We compared the effects of seed predators on pink versus white morphs in both sites using two models, one for probability of infestation, and the other for seeds lost due to predation, i.e., degree of damage. For probability of infestation, we used a binary indicator of infestation status as the response and morph color, site, and their interaction as fixed effects. For the degree of damage, we used the number of intact, endosperm-containing seeds plant as the response. Fixed effects were morph color, site, infestation status, interactions, and the covariate of flowers per head to control for differences in flowerhead size. Source plant was a random effect in both models. Seed counts were modeled with a negative binomial distribution, which is appropriate for count data with overdispersion (Littell et al. 2006). Our analyses of seed predator effects are like those in Carlson and Holsinger (2010) except that here we used seed counts rather than seed set (fertile seeds/flowers per head) because flowers per head was a predictor in subsequent models. All statistical analyses for Question 1were performed in SAS 9.3 in PROC MIXED or PROC GLIMMIX with Kenward-Rogers adjustments to degrees of freedom.

Q2: Are differences in nectar, pollinators and seed predators directly associated with color or a result of correlations with other plant traits?—We used path analysis to assess whether color is directly or indirectly associated with differences in nectar, pollinators or seed predators. Path analysis is appropriate because it makes possible the unraveling of direct and indirect effects across multiple variables, yet because the focus is on statistical associations rather than experimental manipulations, significant effects do not necessarily indicate causal relationships (Mitchell 1993; Shipley 1999). This approach is often used among studies of plant-animal interactions and selection on plant traits, especially in recent decades (Gomez 2000, 2003; Mothershead and Marquis 2000; Hamback 2001; Cariveau et al. 2004). To construct our model, we hypothesized that nectar production, pollinator behavior, seed predation could be directly associated with color, and/or they could be indirectly associated with color through one or more of the following core covariates: plant height, number of heads per plant, mean number of flowers per head, and fresh flowerhead length. Because these correlations were central to the path analysis, we used only the 92 plants for which all four covariates were measured. Plant age was not included in the path analysis because it was strongly correlated with total heads per plant (R=0.63) and height (R=0.86).

Our path model included both continuous and binary variables. To analyse the model we constructed a directed acyclic graph (Spiegelhalter et al. 1996) representing the structural relationships among variables and specified regression relationships among them, using a logistic link for binary response variable. We implemented the model in JAGS v2.1.0 (Plummer 2003) using vague normal priors (mean=0, precision=0.1) on regression coefficients and gamma priors (0.1, 0.1) on variance parameters. We used a multivariate normal prior with mean vector 0 as the prior for the four core covariates mentioned above and a latent logistic variate associated with the binary covariate color. We used a Wishart (I, 7) prior on the precision matrix of the multivariate normal.

We constructed two path analysis models, one full and one reduced, to test the estimate relationships between color, covariates, nectar, pollinators, and seed predation. The full and reduced path models also included relationships with fecundity and seed mass; these components are described in Question 3. In the full model, we simultaneously examined the effects of color, site, color × site and plant traits on each of the following response variables: nectar accumulation by Day 2, the percent of heads infested, the degree of seed predator damage, pollinator visit rates, and visit durations. For pollinator behavior, we used mean hourly visit rate and mean visit duration for each plant as response variables, averaged over AM and PM recording sessions. We included nectar accumulation as an additional fixed effect on both pollinator-related responses. We square-root transformed the response variables of nectar accumulation and pollinator visit duration to meet assumptions of normality. Comparisons involving degree of damage included seed counts in undamaged heads as an additional covariate, to adjust for fecundity differences not associated with predation. In the same path model, we examined the reciprocal correlations between each pair of plant traits or each plant trait and color. We did not examine direct relationships between pollinators and seed predators, because these organisms are unlikely to influence each other directly, given their non-overlap in space and time within flowerheads. For similar reasons, we did not examine direct effects of nectar production on seed predation. Because nectar was measured on only 23 of the video plants, nectar values were imputed as part of the analysis for the remaining plants in the analysis, using the four covariates as well as site and color effects as predictors (Rubin 1976; Yuan 2000; Evans et al. 2010). These imputations made it possible to directly compare nectar to pollinator visits, but they also weakened our ability to detect correlations between nectar, color, and covariates. We therefore included a separate path in the model allowing us to examine these nectar correlations while excluding the imputed data.

The large number of variables in the full model severely limited our statistical power, so we constructed a reduce model and then compared their model fits, using the DIC criterion (Spiegelhalter et al. 2002). The variable reduction technique we chose *a priori* was to eliminate all non-significant variables with credible intervals in which the absolute value of the upper interval was less than twice the absolute value of the lower interval, or vice versa. We retained the four core covariates, and we retained any main site or color effects for which corresponding interaction effects were included. This resulted in the elimination of 34 of the 95 focal comparisons. Because the performance of the reduced model was far superior to that of the full model (DIC of 1557 vs. 1726 respectively), we report only the results from the reduced model.

We were also interested in the effects of two additional traits –specific leaf area (SLA) and leaf chlorophyll content– on color and the focal responses. These two leaf traits could not be included in the full path analysis because of sample size limitations, so we instead ran separate analyses to examine their relationships with other traits, with color, and with two response variables: per-plant means of nectar production and percent infestation. We could not compare leaf traits to pollinator behavior because the datasets did not overlap, nor could we use degree of damage because it was inestimable in all but five plants. We used ANOVAs to explore differences in mean leaf trait values between pink and white morphs. We then preformed two multiple regressions, one each for nectar accumulation and percent infestation as response variables, using the two leaf traits as predictors. Square-root or log transformations were used as needed on response variables to improve normality of residuals. Finally, we assessed all pair-wise relationships between leaf traits and covariates.

Q3: Is floral pigmentation under balancing selection, and if so, is selection direct or indirect via biotic interactions or correlations with other plant traits?— We used the same path analysis to examine plant fitness of pink and white morphs and to explore trait correlations or biotic interactions were involved in favoring each morph. A benefit of path analysis in this context is that its coefficients are equivalent to selection gradients, i.e., the direct selection coefficients independent of trait correlations (Lande and Arnold 1983). We measured fitness with two metrics: potential maternal fecundity–*i.e.*, excluding losses to seed predators– and realized maternal fecundity. Seed mass was included with the fecundity measures because it was previously shown to underlie differences in seed germinability (Carlson and Holsinger 2010).

In this portion of the path analysis model, we examined linkages for seed mass and potential fecundity, but tested only a subset of those for realized fecundity. For seed mass, we examined its effects on biotic interactions, nectar, and potential fecundity, as well as its relationships with site, color, color × site, and the four trait covariates. For potential fecundity, we examined the effects of site, color, color × site, covariates, seed mass, nectar, pollinator visit rates, and visit durations. For realized fecundity, we examined only the effects of potential fecundity, percent infestation and degree of damage. Potential fecundity was log transformed and seed mass was square-root transform to normalize the residuals. We imputed missing data for potential fecundity, seed mass, visit rates, and visit durations as above. Throughout, we used imputed values only when they were predictors in a comparison; data used for response variables were limited to actual measurements.

Outside of the path model, we tested for fecundity differences associated with SLA or chlorophyll content measured in Garcia's Pass only. We compared the effects of both leaf traits on the responses of realized fecundity (log transformed) and seed mass. Comparisons could not be made using potential fecundity because these data were inestimable for over a third of plants with chlorophyll data, due to 100% of their seedheads showing evidence of predation. These tests of fecundity differences were performed in Proc MIXED (SAS 9.3) with color as a random effect.

Results

Q1: Are there differences in nectar, pollinators, and seed predators between colors, sites, and over time

Nectar measurements—The volume of nectar accumulated in *P. aurea* flowerheads was significantly greater in white relative to pink color morphs, and this difference was not specific to either site or day of measurement (Fig 1A; color: $F_{1,78}=6.6$, $P=0.01$; all interactions with color: $F<0.59$, $P>0.45$). There was also more nectar accumulated in flowerheads by Day 4 than by Day 2, but significantly so only in the Garcia's Pass site (day: $F_{1,42}=82.6$, $P<0.0001$; site: $F_{1,85}=33.4$, $P<0.0001$; day × site: $F_{1,39}=42.6$, $P<0.0001$).

Per-flower nectar replenishment rates at Marloth also varied over time, and again, white morphs replenished nectar at a higher hourly rate than did pink morphs (Fig 1B; color: $F_{1,12}$=9.7 P=0.009; day: $F_{3,47}$=9.9, P<0.0001, color × day: $F_{3,48}$=0.23, P=0.87). Mean daily male-phase replenishment (Day 2) was significantly higher than mean daily replenishment in either day of the female phase (Day 3: $F_{1,42}$=2.5, P=0.02; Day 4: $F_{1,58}$=5.4, P<0.0001).

For all accumulation and nectar replenishment rate analyses, the patterns of significance for mass of sugars (results not shown) were equivalent to those observed for volume of nectar. This was not the case for mean concentration of sugars, however, which was not significantly related to color, day, or any interactions for nectar replenishment (F<3.1, P>0.09) or for nectar accumulation (F<1.8, P>0.17). The percent sugars in accumulated nectar differed between sites, however ($F_{1,47}$=11.5, P=0.002). In the nectar accumulation study, sugar concentration was 15.0 ± 0.36 (w/w: mean ± SE, n=44) for Marloth and 17.9 ± 0.48 (n=49) for Garcia's Pass. Percent of sugars in replenished nectar, averaged over the 4 days of measurement, was 13.7 ± 0.4 (n=155) for Marloth.

In the re-analysis of nectar data that included two floral traits as covariates, we found that nectar volumes increased with increasing numbers of flowers per head, as measured by both per-flowerhead accumulation ($F_{1,84}$=5.5, P=0.02) and per-flower replenishment ($F_{1,15}$=4.5, P=0.05). The total length of each flowerhead (from pedicel to style tips) was also positively correlated with nectar accumulation rates ($F_{1,85}$=5.4, P=0.02), but flowerhead length was not detectably related to replenishment ($F_{1,21}$=0.0, P=0.98). For the nectar accumulation model, color was no longer a significant predictor when covariates were included ($F_{1,75}$=0.80, P=0.37). Otherwise, the patterns detected previously for accumulation and replenishment were unchanged. Finally, our test of correlation between the two nectar measures revealed that plants accumulating nectar at higher rates were also replenishing nectar at significantly higher rates ($F_{1,9}$=8.25, P=0.02).

Pollinator preferences—We observed 701 avian pollinator visits to *P. aurea* over 243 hours of video in both sites combined (Table 1). The dominant visitors were sunbirds, responsible for over three-fourths of observed visits, and the most frequently identified among them was the Orange-breasted Sunbird, a Western Cape endemic. The Cape Sugarbird, another endemic, was of lesser importance in both Marloth in Garcia's Pass, making <15% of visits to flowerheads.

In both sites, pollinators made longer-lasting visits to white relative to pink morphs, but the numbers of visits to each morph were similar (Table 2, Fig 2). The increased amount of time spent in white morphs was detected in both the per-plant and per-flowerhead analyses (Figs 2A-B), although color differences at the per-flowerhead level were only significant in Day-1 heads in Marloth (Tukey-adjusted *P*=0.048, Fig 2B). Birds also made longer visits to flowerheads in the afternoon vs. morning recording session (Fig 2A), and they also made longer visits to heads aged to Day 2 vs. Days 3-4, but only for white morphs at Garcia's Pass (Tukey-adjusted *P*=0.03, Fig 2B). The numbers of visits made to flowerheads and plants, in contrast, were not related to flowerhead color or time of recording session, although they were significantly higher in Marloth (Figs 2C-D, Table 2). In Marloth, fewer plants were left unvisited as well: 92% of all recorded plants were visited at least once at Marloth, relative to only 65% at Garcia's Pass. Visit rates to individual flowerheads were also greater to flowerheads aged to Day-2 relative to Days-3-4 (Tukey-adjusted *P*=0.005, Fig 2D), and this could not be attributed to their relative frequency, since Day-3-4 heads were the most recorded in both sites.

Seed predation—*Protea aurea* color morphs did not differ in either the probability or degree of infestation, and this was consistent across both study sites (Table 3). Our analysis of per-seedhead seed counts revealed that infested seedheads contained significantly fewer seeds than did uninfested seedheads. Even so, none of the interactions with infestation status were significant, indicating that there were no site- or color-specific differences in the degree of damage (Table 3).

Q2: Are differences in nectar, pollinators and seed predators directly associated with color or do they result from correlations with other plant traits?

The path analysis revealed that flowerhead color was not directly related to any of the three responses of interest, *i.e.*, nectar production, pollinator behavior, or seed predation. Color was significantly associated with one covariate, however, the number of flowers per head (Fig. 3A, Table S1). In fact, it was this covariate rather than color that showed significant associations with two of the three focal responses: plants with more flowers per head had higher infestation rates and, unsurprisingly, higher nectar accumulation per flowerhead. The correlation with nectar was nevertheless only evident in the path model when imputed nectar data were excluded (Fig. 3A). We did not detect significant associations with nectar (Fig. 3A, Table S1), nor did we detect a direct relationship between pollinator behavior and nectar accumulation rates (Figure 3A). We did, however, detect two additional correlations that involved covariates: (1) plant height was positively associated with the number of flowers per head and (2) plants with shorter heads had higher rates of infestation. Pollinator visit rates and durations were not detectably associated with any variable except that visit rates differ significantly between sites (Fig 3A, Table S1). Although we only present results from the reduced path model, those from the full path model were very similar (Table S2).

In the analyses of leaf trait correlations performed outside the path model, we detected significant correlations involving both leaf chlorophyll content and SLA in Garcia's Pass. White morphs had thinner or less dense leaves (higher SLA; $F_{1,50}$=6.9, P=0.01) and higher levels of chlorophyll, although the chlorophyll difference was only marginally significant ($F_{1,11}$=4.27, P=0.06). Chlorophyll content was also significantly higher in plants with higher rates of nectar accumulation ($F_{1,9}$=5.6, P=0.04), yet SLA did not covary with nectar ($F_{1,9}$=0.19, P=0.67). Neither chlorophyll index nor SLA were significantly correlated with percent infestation (P>0.28). Finally, chlorophyll index and SLA were not significantly related (P>0.10) nor did either covary significantly with any of the four covariates in pairwise comparisons (P>0.15).

Q3: Is floral pigmentation under balancing selection, and if so, is selection direct or indirect via biotic interactions or correlations with other plant traits?

In the selection component of the path analysis, we detected no direct selection on color through differences in seed mass, potential fecundity, or realized fecundity. Instead, each fecundity measure was significantly associated with at least one covariate or biotic interaction and realized selection on color arose as a correlated response (Fig 3B, Table S1). Seed mass was higher in plants with longer flowerheads, and it was higher in plants with higher rates of infestation (Fig 3B). There were also differences in the mean seed mass between sites (Garcia's Pass: 37 ± 0.6 mg, Marloth: 27 ± 0.5 mg). Potential fecundity was positively associated with both the number of heads per plant and the number of flowers per head, and it was negatively associated with seed mass. Realized fecundity, in turn, was strongly positively correlated with potential fecundity, but was negatively correlated with both percent infestation and degree of damage. The link between predation and realized fecundity provides a route by which plants with more flowers per head, –*i.e.,* usually white morphs–, are disfavored by predator-mediated selection (selection coefficient: -0.58; Fig. 3B). Those same plants are nevertheless favored in the absence of predation, through the links between potential fecundity, realized fecundity, and flowers per head (selection coefficient: 0.22). There were no significant links connecting fecundity measures to pollinator behavior or nectar accumulation.

Outside of the path model, chlorophyll index was significantly positively correlated with realized fecundity ($F_{1,10}$=6.45, P=0.03), but it was not detectably related to seed mass

($F_{1,10}$=0.03, P=0.87). Specific leaf area was not significantly correlated with realized fecundity ($F_{1,10}$=0.02, P=0.89) or with seed mass ($F_{1,10}$=2.76, P=0.13).

Discussion

Nectar production and pollinator behavior differ between color morphs and over time

Nectar—Nectar production in our *P. aurea* study populations varied notably between sites, between color morphs, and over time within flowers and heads. Color-specific differences in nectar have not previously been examined in *Protea*, nor, to our knowledge, have they been demonstrated previously in any plant species (c.f. Galen and Kevan 1980; Waser and Price 1981; Stanton 1987; Elam and Linhart 1988; Eckhart et al. 2006). Research on *Protea* nectars has typically focused on the types and composition of sugars (Cowling and Mitchell 1981; Nicolson and Van Wyk 1998; Nicholson and Thornburg 2007) or its energetic value to avian pollinators (Mostert et al. 1980; Calf et al. 2003b) and has largely ignored nectar dynamics within species. Our detailed measurements in *P. aurea* indicate that nectar variability is related to at least three traits or conditions: flowerhead color, the number of flowers per head, and flowerhead age or gender. White flowerheads have higher rates of nectar accumulation, but this is predominantly because they contain more flowers than do pink flowerheads. Nectar replenishment, in contrast, is dependent on both flowerhead color and flowers per head. In other words, heads with higher flower counts not only have more nectaries, which would logically allow for more nectar overall, but they also have higher replenishment rates per nectary within a flower. Such positive associations between nectar production and floral size or display have been observed across a wide range of taxa (Ashman and Stanton 1991; Cresswell and Galen 1991; reviewed in Delph 1996), and in some cases, they may have a genetic component (e.g., Worley and Barrett 2000) as is sometimes shown for nectar variability in general (Zimmerman and Pyke 1986; Hodges

1993; Boose 1997; Vogler et al. 1999; Mitchell 2004). In *P. aurea*, the observed correlations between flowers per head and nectar may in part reflect environmentally-driven differences in plant condition or resource status (Rathcke 1992; Rausher 1992; Campbell 1996; Nicholson and Thornburg 2007), yet given that replenishment rates differ between colors independent of flower counts, pleiotropic effects between color, or some covariate, and nectar are also likely involved.

Daily nectar replenishment in *P. aurea* was not only slightly biased towards white, but it was also greater in the male relative to female phase. Male-biased nectar production has been observed across a wide range of plant taxa (e.g., Bell et al. 1984; reviewed in Cruden et al. 1983; Carlson and Harms 2006), but this is the first suggestion of such a pattern in the Proteaceae. Although gender-biases may be present in other *Protea* species, it is also possible that *P. aurea* is unique, given that its rapid, synchronous flower development within heads is documented nowhere else in the genus (Rebelo, pers. comm). Patterns of nectar accumulation, unlike those for replenishment, provide less clear evidence for nectar biases between gender phases. In Marloth, nectar volumes barely increased between Day-2 and Day-4, whereas in Garcia’s Pass, the volumes more than doubled in both color morphs. Surprisingly, nectar differences between the two sites appeared to be unrelated to plant water status, as might otherwise be expected (Zimmerman 1983; Carroll et al. 2001; Nicholson and Thornburg 2007). Nectar accumulation by Day 4 was far higher in the plants in the drier site, Garcia’s Pass. Because populations were sampled in different years, it is impossible to disassociate year from current weather from genetic effects; even so, genetically-based site differences are possible, given that neutral microsatellite markers show some divergence between these two sites (Prunier & Holsinger, unpublished data).

A comparison of our accumulation and replenishment data suggests that removing nectar actually increases the net amount of nectar produced. If we assume production is constant across all flowers per head, twice-drained flowerheads should produce an estimated volume of 4.0 mL ± 0.81 SE by Day 2, which is far less than the 0.96 mL ± 0.21 that accumulated in Day 2 flowerheads from the same plants at Marloth. The positive feed-back pattern of removal stimulating extra production is often –but not always– observed in nectar studies using similar techniques (Castellanos et al. 2002; Ordano and Ornelas 2004), and in some cases, differences may be associated with nectar reabsorption, as was shown for another member of the Proteaceae (Nicolson 1995). Detailed studies of nectar regulation in *Protea* would be valuable for clarifying the causes and potential costs of elevated production rates following removal.

Pollinators—When nectar rewards differ consistently among plants or over time, as for *P. aurea* color morphs, the foraging preferences of pollinators often match those differences (e.g., Melendez-Ackerman et al. 1997; Jones and Reithel 2001; Carlson 2008). The longer visits that sugarbirds and sunbirds made to white *P. aurea* flowerheads are consistent with nectar differences between morphs, although our subsequent path analysis on per-plant means failed to detect such a link. The higher visit rates and, to a limited extent, visit durations to male-phase heads (Day-2) also indicate that pollinators are tracking rewards on a head-by-head basis. Increased visit rates and visit durations with higher rewards are commonly observed across pollinating taxa (Zimmerman 1983; Melendez-Ackerman et al. 1997; Aizen and Basilio 1998; Carlson 2007; Carlson 2008; but see Lara and Ornelas 2002) and may even form in the absence of visual cues (Miller et al. 1985; Hurly 1996; Irwin 2000). Even so, nectar differences in our study are confounded with differences in color and morphology, such that we cannot rule out the possibility that pollinators are responding something other than nectar. Color preferences are widespread in pollinating animals and insects, even in the absence of known nectar differences (Mogford 1974; Waser and Price 1981; Stanton 1987; Melendez-Ackerman and Campbell 1998; Schemske and Bradshaw 1999). For example, in

Raphanus sativus, nectar production does not differ among color morphs yet honey bees consistently favor white or yellow morphs and syrphid flies prefer pink morphs (Stanton 1987). Although the results of our path model (discussed further below) failed to detect an association between pollinator behavior and color or any other measured floral trait, these findings are quite preliminary; experimental manipulations are required to fully disassociate the effects of floral traits and nectar on pollinator response (e.g., Carlson 2007).

Independent of color and floral phase, *P. aurea* plants in Marloth received three times as many pollinator visits as those in Garcia's Pass. These striking differences cannot be explained within the scope of our study, because our data are confounded by two factors influencing bird behavior: local environment and year of sampling (e.g., Gill and Wolf 1977; Symes et al. 2001; Calf et al. 2003a). We suggest, however, that among the factors that differ between our sites, population size and the presence co-flowering species may be key. The Marloth population was fairly small (~0.25 km^2) with no co-flowering *Protea* species, whereas the Garcia's Pass stand extended in a nearly continuous band for over 10 km and was intermixed with co-flowering *P. neriifolia* and *P. eximia*. As a result, *P. aurea* plants at Garcia's Pass likely experienced high levels of intra- and inter-specific pollinator competition, whereas those at Marloth experienced less competition but were still sufficiently abundant at attract pollinators (e.g., Zimmerman 1981; Campbell 1985; Field et al. 2005). Pollinator competition may be particularly detrimental to *P. aurea* because its nectar rewards are lower than many of its co-occurring congeners, including *P. eximia*, *P. repens,* and bearded proteas *P. grandiceps, P. coronata, P. neriifolia,* and *P. laurifolia* (Calf et al. 2003b, Nicholson and Thornburg 2007). Possibly as a result of nectar differences, Calf et al. (2003a,b) found that Cape Sugarbirds visited bearded proteas more often than *P. aurea* and sister species, even though *P. aurea* and *P. mundii* were more abundant in their study site.

Seed predators—Unlike the current study that revealed no predator biases between colors, our previous work on four *Protea* species showed that seed predators consumed a significantly smaller fraction of seed output in pink relative to white morphs in half of our study populations (Carlson and Holsinger 2010). We suggested that decreased damage on pigmented morphs, when it occurred, was potentially due to differences in secondary chemistry and anti-herbivore compounds (e.g., Fineblum and Rausher 1997; Irwin et al. 2003; Strauss et al. 2004; Johnson et al. 2008; Hanley et al. 2009). We were less able to explain the absence of predator preferences in other sites, although unidentified gradients in seed predator communities or environments may be involved (J. Carlson, unpublished). In both of the current study sites, seed predators did not preferentially attack or consume seeds of white morphs, which is unsurprising for Marloth, since Carlson and Holsinger (2010) found no differences there either. Although preferential attack between colors was not observed and therefore cannot be linked with other traits, we suggest that the trait correlations revealed in Question 2 help illuminate how gradients in predator effects may function across *P. aurea* populations.

Variation in floral display size explains differences in biotic interactions and nectar better than color per se

Although current data show that pollinator behavior (this study), nectar production (this study), and seed predation (Carlson and Holsinger 2010) all differ between *Protea* color morphs, we found that none of these factors were directly associated with color independent of correlations with other traits. For nectar, accumulation rates were directly linked to flowers per head and only indirectly related color. For pollinators, color was a significant predictor of behavior only when covariates were not included, possibly due to reduced statistical power in the path model or to weak associations between pollinator responses and other variables, which may have obscured color effects. For seed predators, infestation rates were higher in plants with shorter flowerheads, higher flower counts, and heavier seeds, but we did not detect any differences directly associated with color. These intriguing correlations with infestation rates provide clues into how *Protea* seed predators choose which plants or flowerheads to attack (see also Gomez 2003; Cariveau et al. 2004; Strauss and Irwin 2004; Caruso et al. 2010;), and they provide an indirect route by which flowerhead color and predation could occasionally be linked. If seed predators target heads that have more flowers (or heavier seeds), but the correlation between color and flower counts (or color and seed mass; c.f. Carlson and Holsinger 2010) varies in strength among sites, then color-specific seed predation would contribute to heterogeneity among sites. Similarly, variation in seed predator communities or strength of preferences may also promote cross-site heterogeneity (Carlson and Holsinger 2010). Such explanations are only possible, however, if correlations between seed or flowerhead size and color are recurrent, due to either costs of pigmentation or a genetically-based trait pleiotropism. Although costs of pigmentation are possible (Steyn et al. 2002), we deem trait pleiotropisms a more probable explanation.

Pleiotropic relationships between floral color and other floral and vegetative traits appear to be common among flowering plants (Armbruster 2002), although the biosynthetic pathways are often not well understood (but see Coberly and Rausher 2003; Coberly and Rausher 2008; Streisfeld and Rausher 2009). In *P. aurea*, we detected differences between color morphs in one floral trait and two leaf traits: the number of flowers per head, SLA (Garcia’s Pass only), and leaf chlorophyll index (Garcia’s Pass only). A reduction in floral display size of pigmented relative to unpigmented morphs has been observed in several other polymorphic species (e.g., Wolfe and Sellers 1997; Gomez 2000; Frey 2007), although the inverse pattern of larger floral displays in pigmented morphs is detected nearly as often (Wolfe 1993; Levin and Brack 1995). Regarding leaf traits, Frey (2007) found that more heavily pigmented plants of *Claytonia virginica* had smaller leaves, but morphs did not differ in maximum photosynthetic rate or stomatal conductance. In *P. aurea*, in contrast, white morphs may in fact have increased photosynthetic capacity, given that their leaves contain more chlorophyll. Nectar was also positively related to chlorophyll index, suggesting that if these plants indeed have higher photosynthetic capacity, they may be allocating the extra photosynthate in part to nectar (Southwick 1984).

The pleiotropic associations detected here likely involve a suite of plant traits, including many we did not measure in this study (e.g., Goplen 1992; Strauss et al. 2004; Coberly and Rausher 2008; reviewed in Strauss and Whittall 2006). For example, the production of anthocyanin in floral and vegetative tissues was correlated in four *Protea* species including *P. aurea* (Carlson and Holsinger 2010). This pattern is known from several other polymorphic species as well (*Ipomoea purpurea*, Schoen et al. 1984; *Clarkia unguiculata*, Bowman 1987). In some cases, pigmentation is also associated with increased plant defense or survival through stressors, likely due to direct effects of anthocyanin or shared biochemical pathways with other compounds (e.g. Koes et al. 1994, Fineblum and Rausher 1997, Warren and Mackenzie 2001, Strauss et al. 2004). Interestingly, the color differences revealed in the current study differ from those detected previously across 10 populations of four *Protea* species (Carlson and Holsinger 2010). Specifically, seed mass –but not flowers per head or SLA– differed between color morphs of four *Protea* species, in contrast to our current results. These discrepancies are in part related the earlier study's focus on cross-species processes, which resulted in reduced sampling within sites and made it difficult to detect differences that were weak or site-specific. We now know that cross-population variation in patterns of color specificity are in fact quite likely in *P. aurea*, given its high potential for genotype by environment effects as well as genetic differentiation among populations (Prunier and Holsinger 2010; Carlson et al. 2011). The morphological differences we observed between color morphs cannot be attributed to environmental effects, however, because first, differences in *Protea* floral pigmentation and leaf traits are heritable (Rourke 1980; Vogts

1982; Coetzee and Littlejohn 2007; Carlson et al. 2011), second, color morphs were spatially intermixed within sites, and third, the pattern involving flower counts was consistent between sites.

Color *per se* is not a target of selection, but a correlated trait is subject to balancing selection through negative pleiotropisms and seed predation

Because floral color is so often correlated with plant traits that directly affect plant fitness, color variation may be maintained through correlational selection instead of direct selection on color *per se* (Armbruster 2002, Strauss and Whittall 2007, Rausher 2008). This appears to be the case in *P. aurea*, given that color was not significantly associated with any fitness measures, yet a trait directly associated with color –*i.e.*, the number of flowers per head– was subject to both positive selection and negative selection. Leaf chlorophyll content was also under positive directional selection via realized fecundity, and this trait was phenotypically linked to color as well. The mediation of selection on floral color via other plant traits has been demonstrated in several polymorphic species. For example, Gomez (2000) showed that white-flowered plants had larger floral displays, which resulted in higher fitness associated with pollinator preferences for larger floral displays. Coberly and Rausher (2003, 2008), found that white morphs had lower fertilization success under stressful conditions, due to a negative trait pleiotropism caused by a mutation that prevents the expression of anthocyanin and associated compounds. Generally, the underlying pleiotropisms that impart differential fitness between morphs are unknown, and there appears to be no consistent pattern across species in which morph is favored (pigmented favored: Waser and Price 1981; Burdon et al. 1983; Wolfe 1993; Levin and Brack 1995; unpigmented favored: Wolfe and Sellers 1997). Within species, the favored morph may depend on the environmental context (Schemske and Bierzychudek 2001, Warren and Mackenzie 2001).

Pollinator behavior was not related to any of our maternal fitness measures, rendering unlikely a straightforward role for pollinator mediated selection in *Protea* color polymorphisms. This aligns with our initial conclusion that avian pollinators were not key selection agents on *Protea* color polymorphisms, based on equivalent levels of pollinator limitation on seed output in pink and white morphs (Carlson and Holsinger 2010). Although pollinator visit durations varied between color morphs in *P. aurea*, pollinator behavior had no detectable effects on long-term maternal fecundity. Male fecundity is also unlikely to differ between color morphs as a result of observed biases, because discrimination was only significant in immature flowerheads. Despite few clear indications so far that pollinators are directly involved in maintaining *Protea* color polymorphisms, our data is still quite limited. For example, pollinator observations from one season per site may poorly represent their responses over multiple seasons, or more importantly, over the lifetime of a plant. Furthermore, the striking, but non-significant time-bias towards white morphs during the male phase at Garcia's Pass hints at the possibility of higher male fecundity for white morphs, assuming a positive relationship between visit length and pollen donation (e.g. Jones and Reithel 2001). Until the strength of pollinator color biases and their consequences to lifetime male and female fecundity are better understood, pollinator-mediated selection cannot be ruled out entirely. Even so, we predict that their contribution should be small at most, particularly relative to that of seed predators and within-site performance differences.

Conclusions

Taken together, our analyses suggest that color polymorphisms in *Protea aurea* populations are promoted by tight, likely pleiotropic, associations between color and key traits affecting seed predation and long-term plant fecundity, namely, the number of flowers per head and leaf chlorophyll content. In congruence with our previous findings on *Protea*, the focal selection pressures acting on these plants include seed predators, which indirectly favor pink morphs, and on-site reproductive differences, which indirectly favor white morphs. These findings provide a unique and detailed view into how *Protea* polymorphisms may be maintained on the local level, and they support a growing body of literature that finds non-pollinating agents and pleiotropic effects to be more important are pollinators in floral color polymorphism maintenance (Schemske and Bierzychudek 2001; Armbruster 2002; Irwin et al. 2003; Strauss and Whittall 2006; Rausher 2008).

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Figure legends

Fig. 1 Nectar volumes of *Protea aurea* from two sites, measured in (a) whole flowerheads after two and four days of accumulation and (b) individual flowers after a 6-h replenishment period from 10 AM to 4 PM each day (Marloth only). Significant main and interaction effects are denoted with * for $p<0.05$ and ** for $p<0.005$

Fig. 2 Time spent feeding (a,b) and hourly visit rate (c,d) on pink or white color morphs of *Protea aurea* in Garcia's Pass and Marloth, based on per-plant (a,c) and per-flowerhead (b,d) analyses. For per-plant comparisons, the time spent per head was averaged across all heads visited, and visit rate was the number of times per hour at least one head was visited prior to departure. For per-flowerhead comparisons, rates and durations were averaged across all same-age flowerheads visited on a plant per day. Bars are raw means ±1SE. Letters in (b) represent significant differences from Tukey-adjusted contrasts. Significant main and interaction effects are denoted with * for $p<0.05$ and ** for $p<0.005$

Fig 3 Significant coefficients from a path analysis that examines whether color is directly or indirectly associated with nectar, pollinators, seed predators, or plant fitness in two populations of *P. aurea*. Although both panels are from a single model, (a) illustrates the effects of color and plant traits on pollinators, nectar and seed predators, and (b) illustrates the effects of plant traits and biotic interactions on seed mass and fecundity measures (i.e., selection gradients). Coefficients for the binary factors site and color are not shown because they are on a different scale. The highlighted path arrows depict routes by which selection could favor each color morph indirectly, based on the significant relationship between color and flowers per head. The asterisks indicate relationships that were only significant in the trimmed nectar plants dataset

Tables

Table 1. Frequency and duration of avian visits to individual flowerheads of *Protea aurea* subsp. *aurea* in two sites in the Langeberg Mountains, Western Cape, South Africa. Two plants (pink and white) were video-recorded for 3+ hours per day in April-May 2010 (Marloth) or 2011 (Garcia's Pass).

Pollinator type	**Garcia's Pass**		**Marloth**	
	Percent of visits	Mean visit duration (sec.)	Percent of visits	Mean visit duration (sec.)
Cape Sugarbird (*Promerops cafer*)	12.5%	48.9	15.0%	35.3
Sunbird species[1]	80.5%	44.3	75.0%	43.4
Cape White-eye (Z*osterops virens* subsp. *capensis*)	7.0%	1.0	8.2%	22.0
Cape Weaver (*Ploceus capensis*)	-	-	1.8%	26.8

[1] Orange-breasted (*Anthobaphes violacea*), Greater Doublecollar (*Cinnyris afer*), Lesser Doublecollar (*Cinnyris chalybeus*), Malachite (*Nectarinia famosa*), and Amethyst (*Chalcomitra amethystina* subsp. *amethystina*)

Table 2. Results of repeated measures mixed and generalized linear mixed models of pollinator visit counts and visit durations to *Protea aurea* in two sites (see also Fig. 2). Plants were recorded for 1.5-2 h twice per day, starting at ~9 AM and 2 PM. Pollinator responses were measured at both per-plant and per-flowerhead levels. For per-flowerhead analyses, flowerhead age (1, 2 or 3-4 days old) was a fixed effect, but AM or PM was random because over half of flowerheads received ≤ 1 visit per day. For per-plant analyses, each bird arrival/departure was counted as only one visit, and visit duration was averaged across all visited flowerheads. Statistically significant effects are bolded.

		Number of visit (offset by hours of video)			Visit duration per flowerhead (sec)		
	Effect	ndf, ddf	F-value	p-value	ndf, ddf	F-value	p-value
Per-plant	Color	1,34	0.42	0.52	**1,69**	**5.72**	**0.02**
	AM or PM	1,81	0.05	0.83	**1,70**	**8.15**	**0.006**
	Site	**1,38**	**12.6**	**0.001**	1,30	2.65	0.11
	Color × AM or PM	1,81	0.04	0.85	1,69	0.03	0.86
	Color × Site	1,34	1.40	0.25	1,69	0.62	0.43
	AM or PM × Site	1,81	0.73	0.39	1,70	2.25	0.14
	Color × Site × AM or PM	1,81	1.52	0.22	1,69	0.12	0.74
Per-flowerhead	Color	1,40	0.81	0.37	1,3	0.00	0.96
	Age	**2,287**	**12.11**	**<0.0001**	**2,158**	**5.27**	**0.006**
	Site	**1,65**	**4.14**	**0.046**	1,49	0.49	0.49
	Color × Age	2,274	0.04	0.96	2,160	1.74	0.18
	Color × Site	1,40	2.21	0.14	1,162	0.61	0.44
	Age × Site	2,287	0.46	0.63	1,158	2.23	0.11
	Color × Site × Age	2,274	0.62	0.54	**2,161**	**3.64**	**0.03**

Table 3. (a) Results of mixed and generalized linear mixed models of the per-seedhead probability of infestation and degree of damage on 92 *Protea aurea* plants total across two sites (Marloth=43, Garcia's Pass=49). Statistically significant effects are bolded. (b) Per-site means are back-transformed LSMEANS ± SE.

a

Response	Effect	ndf, ddf	F-value	p-value
Probability of Infestation	Color	1,205	0.26	0.61
	Site	1,205	0.09	0.76
	Color × Site	1,205	0.29	0.59
Seed count	Color	1,80	1.04	0.31
	Infestation (y/n)	**1,250**	**38.7**	**<0.0001**
	Site	1,79	2.70	0.10
	Color × Infestation	1,250	0.88	0.35
	Color × Site	1,78	2.82	0.10
	Infestation × Site	1,250	1.34	0.25
	Color × Site × Infestation	1,250	0.65	0.42
	Flowers per head	**1,178**	**13.41**	**0.0003**

b

Mean ± SE	Marloth	Garcia's Pass
Probability of infestation	51 ± 6	49 ± 6
Seeds per head, infested	2.8 ± 0.5	2.1 ± 0.4
Seed per head, uninfested	7.5 ± 1.2	4.0 ± 0.7

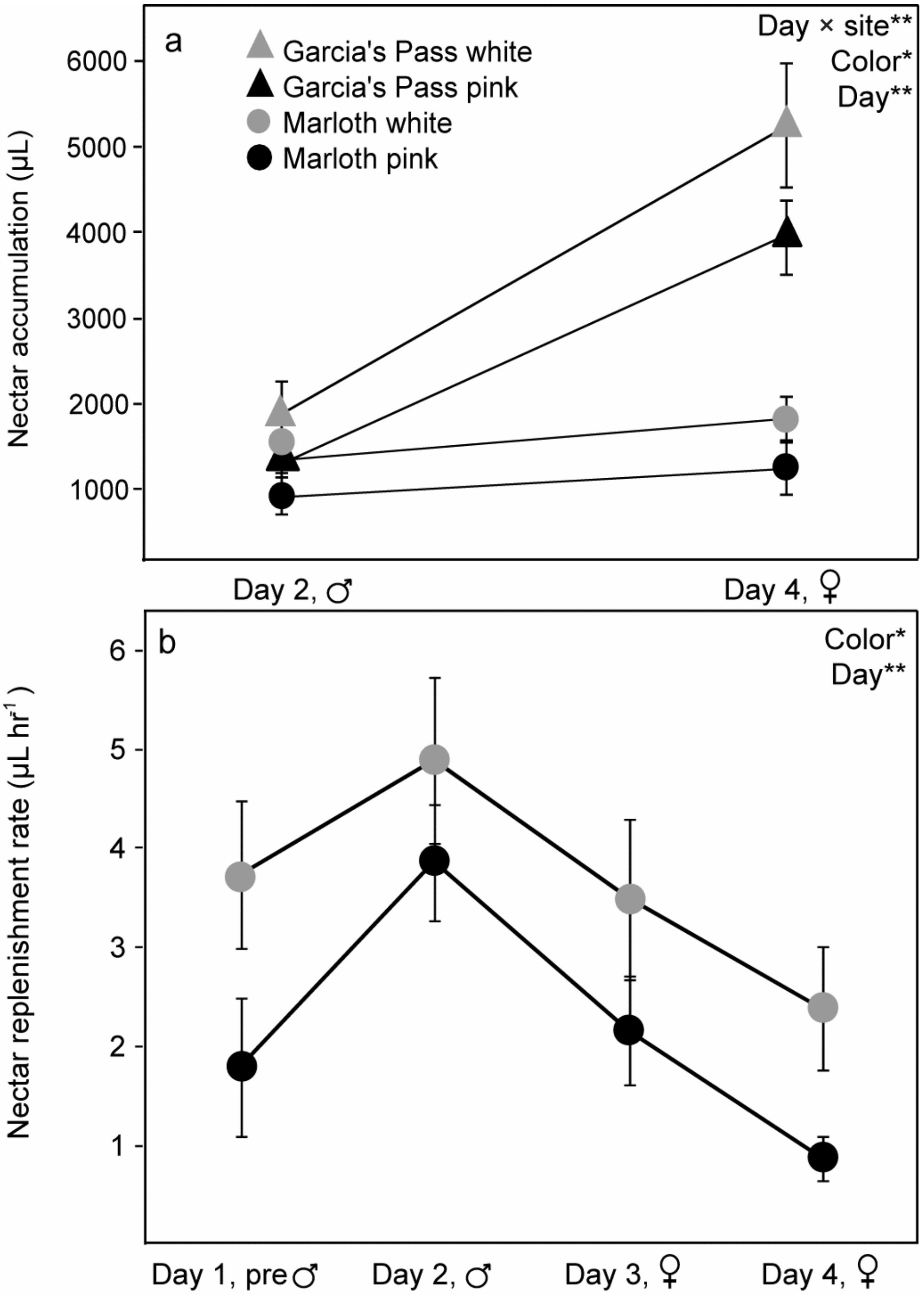
a
Garcia's Pass white
Garcia's Pass pink
Marloth white
Marloth pink
Day × site**
Color*
Day**
Nectar accumulation (μL)
6000
5000
4000
3000
2000
1000
Day 2, ♂
Day 4, ♀
b
Color*
Day**
Nectar replenishment rate (μL hr⁻¹)
6
5
4
3
2
1
Day 1, pre ♂
Day 2, ♂
Day 3, ♀
Day 4, ♀

Fig. 1

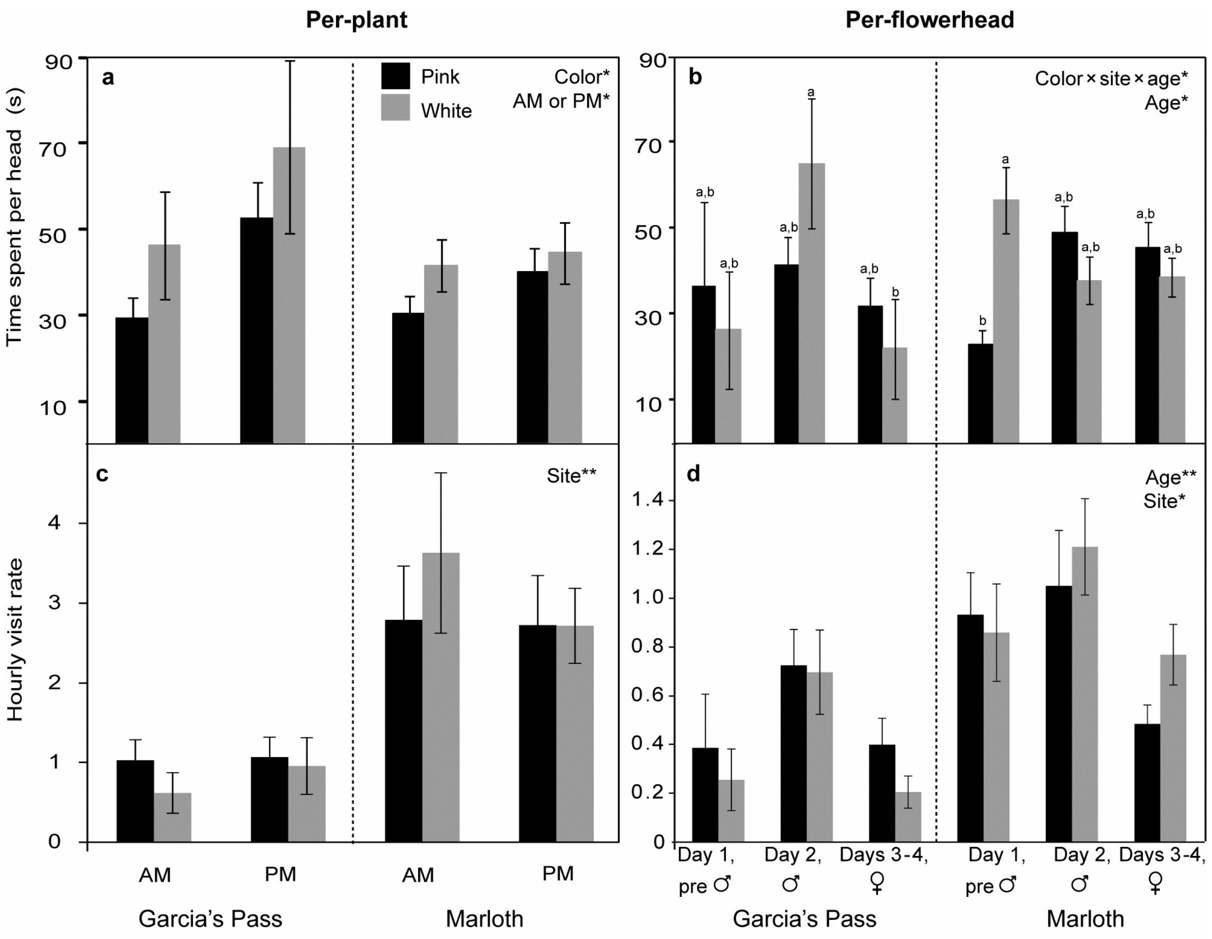
Per-plant
Per-flowerhead
a
b
c
d
Pink
White
Color*
AM or PM*
Color × site × age*
Age*
Site**
Age**
Site*
Time spent per head (s)
Hourly visit rate
90
70
50
30
10
4
3
2
1
0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
a,b
a
b
AM
PM
Day 1, pre ♂
Day 2, ♂
Days 3-4, ♀
Garcia's Pass
Marloth

Fig. 2

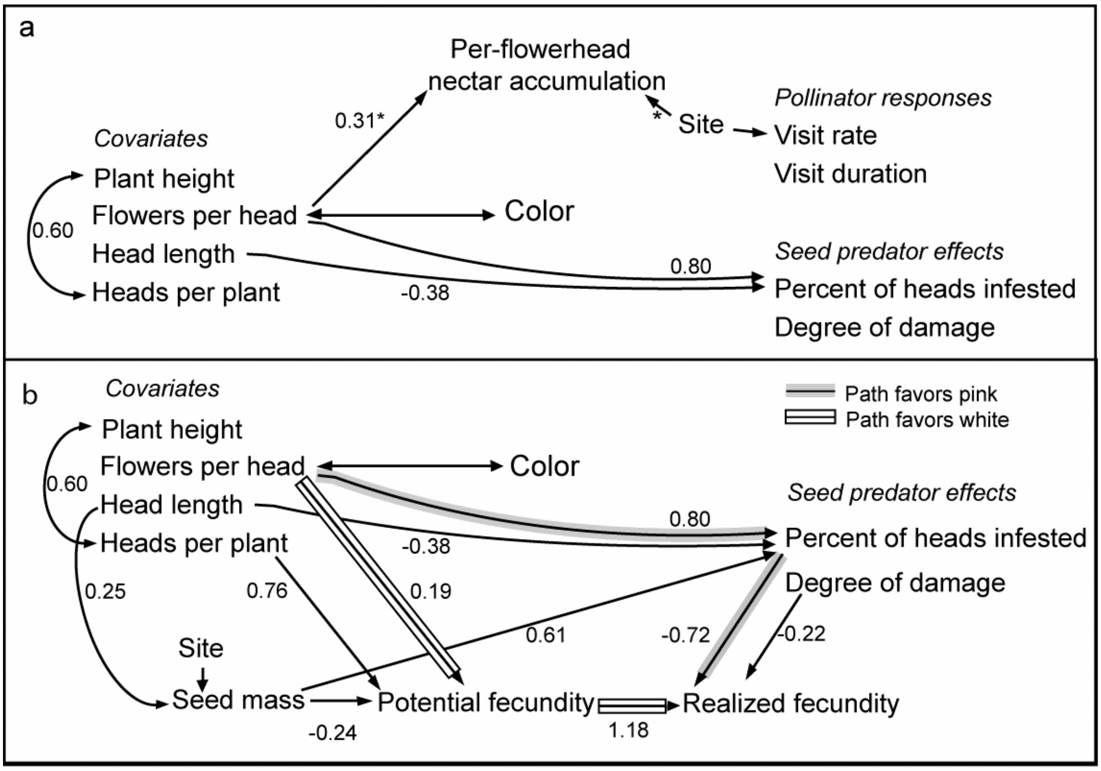
a
Per-flowerhead
nectar accumulation
0.31*
*
Site
Pollinator responses
Visit rate
Visit duration
Covariates
Plant height
Flowers per head
0.60
Color
Head length
Heads per plant
0.80
-0.38
Seed predator effects
Percent of heads infested
Degree of damage
b
Covariates
Path favors pink
Path favors white
Plant height
Flowers per head
0.60
Color
Head length
Heads per plant
Seed predator effects
0.80
-0.38
Percent of heads infested
Degree of damage
0.25
0.76
0.19
0.61
-0.72
-0.22
Site
Seed mass
Potential fecundity
Realized fecundity
-0.24
1.18

Fig. 3