POLLINATION BIOLOGY OF THE MUSHROOM-MIMICKING ORCHID GENUS *DRACULA*

by

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A DISSERTATION

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DISSERTATION ABSTRACT

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Dracula orchids are hypothesized to rely on mushroom mimicry for pollination. These orchids look and smell like mushrooms and are pollinated by mushroom-associated flies in the family Drosophilidae. *Dracula* includes over 130 species, representing a significant radiation, yet there has never been a systematic study of their pollination biology. Elucidating the processes and mechanisms of pollination in these flowers will broaden our understanding of mimicry within the Orchidaceae, a family well known for its diverse pollination strategies, as well as add to the growing literature on the evolution and maintenance of communication signals. In this study we demonstrate the co-occurrence of the mimics and the putative mushroom models, which is important for evolution by natural selection. We also showed that the resemblance to mushrooms is in fact adaptive, a requisite for floral mimicry. We did this by determining that insect visitors are required for pollination and subsequent fruit set with a hand pollination experiment. We also measured increased visitation rates to the orchids when adjacent to mushrooms.

The mechanisms whereby plants attract pollinators can be diverse and often multi-modal, particularly in deceptive systems. *Dracula* orchids are no exception, with both visual and olfactory signals contributing to the overall success in attracting visitors. We

used a series of experiments, first selectively masking the visual and olfactory cues successively, and then using 3D-printed artificial flowers to further disentangle these cues and determine their effect in combination. Upon confirmation that both play a role, we dissected each aspect further. We utilized the artificial flowers to determine the roles of color, contrast, and pattern and employed gas chromatography-mass spectroscopy to identify the volatile signals. The results show that fine-scale contrast is critical to the visual component and that these flowers produce the volatile 'mushroom-alcohol' (1-octen-3-ol) in their labella.

Finally, we specifically address the hypothesis of brood-site mimicry by using a combination of field observations, insect collections, and rearing studies. The flies gain shelter, a rendezvous location, and food from the flowers. However, no mushroom visiting flies hatched from the flowers, suggesting this may be a brood-site mimicry.

This dissertation includes previously unpublished co-authored material.

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Molly Policha 1915-2009

Janet Pasluk Falkenberg 1934-2013

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CHAPTER I

INTRODUCTION

Mimicry, the adaptive resemblance of one organism to another, is one of the most compelling examples of natural selection (Darwin 1859, Bates 1862, Wallace 1870, Poulton 1890, Fisher 1930, Gilbert 1983). Mimicry has long fascinated biologists, with the phenomenon first being recognized in systems of predator evasion in animals. (Bates 1862, Müller 1879). While mimicry has been studied extensively in animals, there have been far fewer examinations of mimicry in plants (Roy and Widmer 1999). Floral mimicry and deceptive pollination have been recognized for over 200 years (Sprengel 1793), but the details of the perceptual biases involved in the evolution of floral mimicry remain an active field of investigation (Dafni 1984, Schiestl 2005, Jersáková et al. 2006, Schaefer and Ruxton 2009, Vereecken and McNeil 2010). Both major types of mimicry found in animal systems, Müllerian and Batesian, can be seen in plant systems as well (Dafni and Ivri 1981, Dafni 1984, Johnson 1994, Roy and Widmer 1999, Gigord et al. 2002, Schiestl 2005, Jersáková et al. 2006, Smithson 2006, Schluter and Schiestl 2008, Ellis and Johnson 2010, Gaskett 2011, Papadopulos et al. 2013). Mimicry is particularly well developed in the Orchidaceae (~1/3 of species), leading some authors to suggest that it has played an important role in the rapid evolution and radiation of the family (Van der Pijl and Dodson 1966, Cozzolino and Widmer 2005).

Müllerian mimicry in flowers requires the convergence of two or more species on a single phenotype, and is selected for by the fitness benefits of increased pollinator visitation for both co-models (Ridley 1996, Roy and Widmer 1999). Conversely, Batesian floral mimicry entails the deceptive exploitation of a previously established

mutualism (Roy and Widmer 1999). This may be between a pollinator and a rewarding plant (Dafni and Ivri 1981, Roy 1993, Johnson 1994, 2000, Johnson et al. 2003), or the sexual deception of male insects seeking female mates (Dafni 1984, Dafni and Calder 1987, Blanco and Barboza 2005, Gaskett et al. 2008, Gaskett 2011).

In orchids, deceptive pollination strategies are apparently successful, given that they have evolved multiple times (Van der Pijl and Dodson 1966, Cozzolino and Widmer 2005). Within the Orchidaceae there are a number of different kinds of mimicry, which play on the perceptional biases of the pollinators. These range from generalized food deception in which nectar-less orchid flowers look similar to other species with nectar, to brood-site mimicry systems where the orchid flowers appear similar to a substrate in which insects oviposit, to pseudocopulation systems in which the orchid flowers both look and smell like female insects and are pollinated by males of the species through an attempt to copulate (Dafni 1984, Jersáková et al. 2006). Specialization may be one of the keys to this success: Adopting a novel pollination strategy may open a new niche, possibly stimulating adaptive radiation (Johnson 2010).

An unusual example of putative fungal mimicry is found in the orchid genus *Dracula* Luer. *Dracula* orchids exhibit a peculiar morphology of the labellum (the modified lower petal) that appears to mimic the reproductive surfaces of gilled mushrooms (Vogel 1978, Dressler 1990, Luer 1993, Christensen 1994, Behar 1995, Jersáková et al. 2006). Many of these orchids also produce volatiles common to fungi (Kaiser 1993b, Kaiser 1993a, Kaiser 2006). This combination of unique floral traits is hypothesized to function in *Dracula* for pollination by fungus gnats seeking brood-sites (Vogel 1978, Christensen 1994). The *Dracula* lineage has indeed been successful,

numbering at least 120 named species (Meyer and Cameron 2009) despite the possible extinction of endemics (Koopowitz et al. 1993), and new species are still being described (Doucette 2011).

Dracula species are generally thought to be fly-pollinated (Van der Pijl and Dodson 1966, Pridgeon et al. 2001, Van der Cingel 2001), and it is one of only a few orchid genera suspected to imitate mushrooms, thereby attracting mushroom-associated flies (Jersáková et al. 2006). Flies (Diptera) have been pollinating plants for a long time, possibly since before the radiation of angiosperms (Labandeira 1998, Ren 1998). Legitimate fly pollination (myophily) is now widespread across angiosperms (Larson et al. 2001). Flies are also exploited in various ways to act as pollinators in deceptive scenarios, including brood site mimicry (Burgess et al. 2004, Van der Niet et al. 2011, Jürgens et al. 2013), yeast mimicry (Goodrich et al. 2006, Stokl et al. 2010), and sexual deception (Johnson and Midgley 1997, Blanco and Barboza 2005, Ellis and Johnson 2010, Gaskett 2011). Due to their ubiquity, flies represent an important and available source of pollination services for plants, particularly in areas with depauperate anthophilous insect communities (Larson et al. 2001, Ssymank et al. 2008). In moist, shady habitats such as tropical cloud forests, which are typically poor in anthophilous insect fauna but rich in mushroom-associated taxa, the evolution of pollination by these mushroom-associated insects should be favored (Mesler et al. 1980). Yet the attraction of fungal-associated dipteran taxa is exceedingly rare within the Orchidaceae and, with the exception of the sexually deceptive system described by Blanco and Barboza (2005), remains largely anecdotal (Jones 1970, Lehnebach et al. 2005, Patterson et al. 2010, Ren et al. 2011, Kelly et al. 2013). Can the success of *Dracula* orchids be attributed to their

imitation of mushrooms and the successful recruitment of mushroom associated flies?

This dissertation focuses on the hypothesis of mushroom mimicry by *Dracula* species by asking three specific questions: Is the resemblance to mushrooms adaptive? How is the deception achieved? What resources are the flies getting from the mushrooms and the orchids (or not)?

STUDY SYSTEM

The mimics: Dracula *spp.*

The genus *Dracula* Luer (Epidendroideae, Epidendreae, Pleurothallidinae) was segregated from *Masdevallia* Ruiz & Pav. partly on the basis of the distinctive mushroom-like morphology of the labellum (Luer 1978). *Dracula* orchids (~125-150 spp.) are restricted to montane or submontane habitats of the Neotropics (Luer 1993). They are epiphytes in mature forests ranging from southern Mexico (1 sp.) to Peru (1 sp.), reaching their peak diversity in the wet forests that cover the slopes of the western Andes in Colombia (>60 spp.) and Ecuador (>45 spp.) (Luer 1993, Jorgensen and León-Yanez 1999).

Some species of *Dracula* have widespread ranges (e.g., *D. vespertilio,* found from Nicaragua to Ecuador), while most are locally endemic (Luer 1993). The plants are epiphytic and occur only in undisturbed, primary cloud forests with high humidity and indirect sunlight (Luer and Escobar 1988, Luer 1993).

Most species flower throughout the year with flowers presented singly, on a successively few-flowered raceme, but some are known to flower only once a year (Luer 1993, L. Jost, pers. comm. pers. obs.). At our study site Reserva Los Cedros, at least four abundant species flower during the rainy season, particularly January through March.

One of the species, *D. felix,* is common, flowers abundantly, and often has many (>20) flowers present at the same time borne on individual peduncles. *Dracula* orchids are epiphytic, but they are frequently found on the ground where they have fallen, or on branches and trunks within 3m from the ground, singly or in clumps (pers. obs. and C. Luer, pers. comm.).

Artificial hybrids between species of sister genera (*Dracula* x *Masdevallia* = *Dracuvallia*), and between species of *Dracula* are common in the horticultural industry (www.ecuagenera.com), yet most species of *Dracula* are distinct and morphologically stable in nature (Luer 1978, Luer 1993). One species, *D. xenos,* is putatively a natural hybrid of a *Dracula* x *Masdevallia* cross, but was treated as a separate, monotypic subgenus by morphological classifications and represents an exceptional case for the genus (Pridgeon et al. 2001). These observations suggest that there are few, if any, post-zygotic barriers to reproductive isolation in nature and pre-zygotic isolating barriers such as pollinator specificity, or phenology are probably the dominant forces of reproductive isolation and speciation in *Dracula* spp. growing in sympatry [see Figures 2.1, 3.1, and 4.3, as well as Supplemental Figures S3.1 and S4.1 (Appendix A) for examples of the diversity within the genus].

The models: Agaricomycotina

It is in part the elevated, radiating veins coursing through the epichile of the labellum that make *Dracula* orchids distinctive (Luer 1978), and indeed this part makes them visually similar to gilled mushrooms (Vogel 1978). In a survey of the mushroom community at our study site in 2011 (unpub.) we found that the majority (62%) of the 1,953 mushrooms encountered were gilled mushrooms or agarics, followed by clavarioid,

bracket, poroid-stipitate, corticioid, puffball, and birds' nest forms of fruiting bodies. Further enhancing *Dracula's* likeness to these mushrooms, the majority (68%) had white gills, and over ¼ of pileate fungi had a white pileus.

The dupes: Zygothrica & Hirtodrosophila *spp.*

The most common visitors to *Dracula* species in western Ecuador have been identified as flies in the family Drosophilidae (Endara et al. 2010). While the pollinators were speculated to be 'fungus gnats' (Vogel 1978, Dressler 1990, Luer 1993, Christensen 1994, Behar 1995, Kaiser 2006) they are in fact drosophilids, and as such are not closely related to either of the fly groups commonly referred as fungus gnats (i.e., the Mycetophilidae and Sciaridae, (Grimaldi and Engel 2005). The most common genera from *Dracula* orchids and the co-occurring mushrooms are *Zygothrica*, and *Hirtodrosophila.* [See Supplemental Figures S4.3a and S4.3b (Appendix A)].

Zygothrica (Grimaldi 1986) and *Hirtodrosophila* (Grimaldi, pers. com.) commonly utilize mushrooms at some stage in their life cycles, which is consistent with the hypothesis that the unique floral traits of *Dracula* flowers function as imitations of fungi to attract these taxa. However, the relationship between these flies and the mushrooms at which they aggregate is not clear. Ironically, most brood site records for *Zygothrica* are actually from flowers (Grimaldi 1987, dos Santos and Vilela 2005), while some species have been seen grazing spores from the reproductive surfaces, and many utilize the mushroom caps for exhibiting mating behaviors (Grimaldi 1987).

The effects of each of these activities on fungal fitness are unknown. Using mushrooms as brood sites may be commensal if the mushroom fitness is not affected by the fly larvae, but it may also be parasitic if the larvae cause substantial damage (Corner

1972, Hackman and Meinander 1979, Bruns 1984, Hanski 1989). Grazing can be considered parasitic if all of the consumed spores are destroyed, but may be mutualistic if some are expelled intact, and the insect acts as a dispersal vector (Lilleskov and Bruns 2005). Using mushrooms as sites for displaying mating behavior is most likely commensal. To date, there are no unequivocal data on what resources or rewards these flies may be seeking at *Dracula* flowers. Although the insect-mushroom relationship may range from casual (commensal) to obligate (parasitic/mutualistic), it is strong enough in at least some *Zygothrica* to be successfully exploited by more than 120 species of *Dracula* orchids. It should be noted that the association of *Zygothrica* with *Dracula* is probably widespread, and not restricted to the area in Ecuador where these studies have taken place. There are unidentified *Zygothrica* specimens in the AMNH collected from *Dracula* flowers in Colombia and Panama (Grimaldi, pers. com.).

Study site

The fieldwork was performed at Bosque Protector Reserva Los Cedros, which is located between 1,250 and 2,200 m elevation on the western slope of the Andes in northwestern Ecuador (00°18'31.0"N, 78°46'44.6"W). This private reserve protects 6,900 hectares of montane cloud forest, 5,800 hectares of which is primary forest. The reserve is a buffer zone for the 300,000 hectare Reserva Ecológica Cotacachi-Cayapas, and is part of the Chocó phytogeographical zone, recognized as one of the most biologically diverse habitats on earth (Myers et al. 2000). The forest canopy here reaches to ~25m and supports abundant mosses and vascular epiphytes. Average canopy cover is ~80%, with an estimated 300 tree species per hectare based on the collection of 2,744 individual specimens of 337 tree species, from 40 genera and 61 families. The five most

common families of trees are Urticaceae (18%), Lauraceae (14%), Melastomataceae (9%), Rubiaceae and Moraceae (6%) (Peck et al. 2008). The reserve experiences an average annual rainfall of 2,903 mm (SE=186.41) (José DeCoux pers. com.). Our studies were conducted in 2008, and 2010-12, during the local rainy season (January-March) when the *Dracula* orchids were in peak bloom.

Reserva Los Cedros is dedicated to sustainable ecotourism that benefits the local community and to developing intercultural collaborations between visiting scientists, and Ecuadorian students and researchers. This kind of coordination is critical to long-term preservation of pristine habitat and the promotion of productive collaborations between international research institutions.

DISSERTATION RESEARCH

Due to the size and scope of this project, my work has very much benefitted from working closely with collaborators, experts and assistants. Several of these individuals are included as co-authors on the articles that form the basis of my dissertation chapters and are mentioned below as I introduce each chapter.

Fieldwork and specimen collection generally involve a variety of paperwork and procedures. This can be complicated when international borders are involved. Language and cultural barriers, when they exist, certainly add their own complications. In addition to the research presented here, I played a pivotal on-the-ground role, spending months in Quito, to procure necessary permits to carry out field work in Ecuador and to export all of our specimens through the Ministerio del Ambiente de Ecuador (No. 001-07 IC-F-DRCI-

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Chapter II

My first research-based chapter establishes a potential fitness benefit to *Dracula* from the resemblance to mushrooms. The title is 'Where *Dracula* lurks: Context drives mimicry in a Neotropical orchid,' and it is co-authored by Rocío Manobanda (Universidad de Los Andes, Venezuela) as well as the co-PIs on the project Bryn T.M. Dentinger and Bitty A. Roy. It has been submitted to the journal *Ecology*. We address four requisites for establishing a true mimicry system whose evolution is driven by natural selection: 1. We confirm with vouchered specimens that the putative mimics (*Dracula* orchids) and models (mushrooms) co-occur. 2. Using a hand pollination experiment we show that *D. felix* requires pollinators for seed set. 3. We document that the same individual insects move between the models and the mimics, and 4. We provide support for the adaptive significance of the plant's mushroom phenotype both by documenting higher insect visitation and fruit set in *Dracula* species relative to the closely related genus *Masdevallia* and by experimentally showing that visitation to orchids is higher when mushrooms are in close proximity.

We found that the attractiveness of floral display was dependent on the density of blooms, and that both large groups of flowers and mixtures of flowers and mushrooms were able to recruit more insect visitors than single flowers on their own. The fact that visitations increased both in the presence of mushrooms and amongst an abundant floral display suggests that the drosophilid visitors may not be discriminating between these two resources. These results support the hypothesis that *Dracula* orchids derive a fitness

benefit from appearing as mushrooms when mushrooms are present in the environment. Our data suggest that these plants may utilize a mushroom-mimicking phenotype to overcome the problems of density-dependent pollinator visitation in a Neotropical cloud forest by exploiting the underutilized resource of mushroom-associated flies as pollinators where conditions favor a constant supply of mushroom fruiting bodies and their associated insects.

Chapter III

In chapter III we address the mechanisms whereby the attraction to Dracula flowers is realized. In this chapter I had the pleasure of collaborating with Melinda Barnadas (Magpie Studios), a phenomenal artist who produced life-like model flowers that we deployed in the field. This chapter also would not have been possible without the generous support of time and energy lent by scent chemist, ethologist, and dissertation advisory committee member Rob Raguso (Cornell University). Both M. Barnadas and R. Raguso are co-authors on this chapter, as well as Aleah Davis (University of Oregon), Bryn Dentinger, and Bitty Roy. It is titled 'Disentangling visual and olfactory signals in mushroom-mimicking *Dracula* orchids using realistic 3-D printed artificial flowers'

As fungal mimicry in *Dracula* is unique within the orchid family, we asked to what extent visual and olfactory aspects of floral phenotype are responsible for attracting drosophilid pollinators. To tease apart the impacts of visual and olfactory cues on pollinator behavior, we performed manipulative experiments in which we deconstructed flowers, reconstructed flowers, built chimeras, deployed artificial flowers, and analyzed the volatile chemistry. The artificial flowers were critical to decoupling sensory aspects of the unique *Dracula* phenotype.

In the first set of experiments we selectively removed aspects of the signaling phenotype by covering the flowers with green-dyed cotton bags (eliminating visual cues) or with airtight plastic bags (eliminating olfactory cues). Next, we used life-like silicone replicas of the flowers and presented either a visual-only fabrication or the fabrication with added odor extracts (extracted from real flowers) to re-create both aspects of attraction. Then we combined the fabricated flower parts with real flower parts creating chimeras that we could use to assay the relative contributions of specific floral regions. Finally, we utilized a series of fabricated flowers displaying different levels of color, contrast and pattern to understand the specific role of the visual cues. These field experiments showed that floral volatiles played a decisive role in insect attraction. GCMS analysis of the *Dracula* flowers (dissected into parts) as well as co-occurring mushrooms and sister-group pleurothallid orchids, demonstrate novel fungal volatile production by *Dracula* orchids.

Floral deconstruction/reconstruction experiments show that both visual and olfactory aspects of floral display are important, while the chimera experiment demonstrates significant contributions from the calyx as well as the labellum. When we teased apart the visual contribution of the calyx we found that contrast was paramount, with pattern playing an important role as well. The solidly colored treatments were the least attractive. When we scrutinized the odor profiles of the *Dracula* flowers, co-occurring mushrooms and the sister-group orchids, we found that the *Dracula* bouquet overlapped with that of the mushrooms and that these fungal scents were novel within the genus. A finer dissection revealed that it was primarily the mushroom-looking labellum that produced the fungal volatile 1-octen-3-ol.

These experiments indicate that both the morphological and olfactory aspects of floral phenotype contribute to the striking evolution of mushroom mimicry in Dracula, and that they act in concert to attract dipteran pollinators in a fungal-rich cloud forest habitat.

Chapter IV

Chapter IV is titled 'Does *Dracula* appear also as a mushroom? Substrate utilization by cloud forest drosophilid flies.' In this chapter we determine who the visitors are and what they are doing. David Grimaldi's (American Museum of Natural History) expertise with the pollinator taxa involved made for invaluable contributions. His morphological assessments were supported by DNA barcoding, in which Ashley Ludden (University of Oregon) played a substantial role. They, as well as Adrian Troya (Escuela Politécnica Nacional, Ecuador) are co-authors on this chapter, as are Rocío Manobanda, Bryn Dentinger, and Bitty Roy.

In this chapter we test the brood-site hypothesis by determining who the visitors are to both mushrooms and orchids, and where they breed. We specifically address this hypothesis using a combination of field observations, collections at flowers and mushrooms as well as malaise traps, and rearing studies followed by morphological and molecular analyses to determine identities and multivariate statistics to examine host use.

Dracula orchids do attract many of the same fly species and similar communities of flies as mushrooms. The same individual flies do move between *Dracula* spp. and mushrooms. Drosophilid visitors show non-random host use amongst substrates (i.e., species specific preferences). Visitors to mushrooms, as well as *D. felix* and *D. lafleurii*, display similar patterns of behavior across the three substrates. Flies are rearing young

from mushrooms and *Dracula* orchids, but different species are breeding on each substrate, and at different frequencies, with the mushrooms being the preferred brood-site.

The brood-site mimicry hypothesis is supported by our data. Flies that otherwise breed in mushrooms are spending time on *Dracula* flowers, where they move pollinia, but do not breed. These flies are still obtaining rewards from their visitation -- mating site, shelter, food, etc. so the deception may not translate to a total loss of fitness, placing their relationship somewhere on the continuum between Batesian (deception) and Müllerian (convergence) mimicry.

Our results also suggest a bi-modal attraction strategy by the flowers with over half of the visitors belonging to fungal associated taxa, while the remainder appear to be specialists, either visiting exclusively one *Dracula* species or visiting more than one *Dracula* species, but not mushrooms. Some of these flower specialists have been caught with pollinia attached. This may suggest that the flies found only on *Dracula* are in fact getting enough of a reward, even without rearing success, to stabilize this relationship.

Chapter V

In chapter V, I summarize the results from chapters II-IV, draw conclusions on the pollination biology of *Dracula* orchids and suggest implications for both mimicry theory and biodiversity conservation.

CHAPTER II

WHERE *DRACULA* LURKS:

CONTEXT DRIVES MIMICRY IN A NEOTROPICAL ORCHID

Bitty Roy designed the experiment in which mushrooms were moved into proximity to flowers. Bitty Roy, Bryn Dentinger and I designed of the rest of the experiments contained herein. Rocío Manobanda helped design the experiment in which we moved whole plants of *Dracula lafleurii*. We all collected data in the field, and I performed the statistical analyses and wrote the chapter with editorial input from these co-authors.

INTRODUCTION

Mimicry has long fascinated biologists, with the phenomenon first being recognized in systems of predator evasion in animals (Bates 1862, Müller 1879). Mimicry is also employed by many plant species, although typically as a mechanism of pollinator attraction (Roy and Widmer 1999, Jersáková et al. 2006). There are two main types of mimicry: 'Batesian,' where the model is well defended (animals) or rewarding (plants) and the mimic is not; and 'Müllerian,' where a number of species converge on a common phenotype. The similarity of two organisms is not, in and of itself, sufficient to establish true mimicry: what sets mimicry apart from superficial resemblance is the role of natural selection in its evolution. While the adaptive nature of mimicry in animals has received copious attention, the same is not true of plants. In their 1999 paper, Roy and Widmer outlined a framework for testing hypotheses of floral mimicry that extends

beyond superficial similarities to establish that resemblance conveys a reproductive advantage to the mimic. Requisites to establishing true mimicry include: 1. overlapping distribution and phenology of the mimic and the model to ensure spatial and temporal interaction for long enough for evolution to occur, 2. the plant in question must require pollinators for seed set (i.e., it cannot be autogamous), 3. the same individual pollinators must move between the mimic and model, and most importantly, 4. the similarity must be important for fitness (Roy and Widmer 1999).

No other plant family boasts as many species that employ some form of mimicry or as many types of mimicry as the Orchidaceae (Roy and Widmer 1999, Cozzolino and Widmer 2005, Jersáková et al. 2006), but many examples remain poorly documented or anecdotal. One peculiar case of putative fungal mimicry in orchids is the genus *Dracula* in which the flowers look and smell like gilled ("agaricoid") mushrooms (Vogel 1978, Luer 1993, Kaiser 2006).

Putative fungal mimicry or at least pollination by 'fungus gnats' (Mycetophilidae and Sciaridae) or other fungus-associated flies (e.g., Platypezidae) has been described in or hypothesized for several plant species (Vogel 1978, Ackerman and Mesler 1979, Mesler et al. 1980, Vogel and Martens 2000, Goldblatt et al. 2004, Okuyama et al. 2008, Ren et al. 2011). Adaptations to attract these mycophilous insects appear to have evolved independently several times within the angiosperms (Ackerman and Mesler 1979, Okuyama et al. 2008, Goodrich and Raguso 2009). It has been suggested that moist, shady habitats, such as cloud forests that are typically poor in anthophilous insect fauna, are rich in mushrooms and mushroom visiting insects, thereby favoring the evolution of pollination by these insects (Mesler et al. 1980).

While it has often been suggested, evidence for mushroom mimicry is exceedingly rare, with only a handful of potential cases. In the Orchidaceae, pollination of the Australian helmet orchid (*Corybas diemenicus*) by mycetophilid flies associated with *Dermocybe* spp. (Jones 1970) is one such case, while in the *Cypripedium fargesii* system, the pollinator (*Agathomyia* sp.; Platypezidae) typically feeds on conidia of an ascomycetous fungal pathogen (*Cladosporium* sp.), rather than being associated with mushrooms (Ren et al. 2011). Additionally, there are cases where putative mimicry turns out to more closely resemble a reward-based pollination system. The twayblade orchid (*Listera cordata*) is pollinated by fungus gnats (Mycetophilidae and Sciaridae), but they receive a nectar reward as opposed to being deceived (Ackerman and Mesler 1979). However in none of these examples is the resemblance to a fungal model so well developed as it is in *Dracula,* whose labella are superficially indistinguishable from small, gilled mushroom caps and produce fragrant mushroom volatiles (Luer 1993, Kaiser 2006).

The genus *Dracula* was segregated from *Masdevallia* in the late 1970's based, in part, on the unique morphology of its labellum, whose "elevated, radiating veins" are reminiscent of mushroom gills (Luer 1993) (see for example, Figure 2.1a). This unusual phenotype led to speculation that flowers in this genus would be pollinated by mushroom-associated flies (Vogel 1978). In support of this hypothesis, Kaiser (1993, 2006) characterized the volatiles produced by *D. chestertonii* as including the 8-carbon alcohols and ketones typical of fungal aromas. More recently, Endara et al. (2010) described the pollination mechanisms for *D. felix* and *D. lafleurii*, implicating mushroom-associated flies in the family Drosophilidae as the pollinators (Figure 2.1c, for example).

While these previous studies all strongly suggest that *Dracula* are mushroom mimics, no rigorous test of this hypothesis has been conducted.

We establish that insect visitation is required for fruit set in *Dracula felix*, and that the similarity to mushrooms confers a fitness advantage by increasing visitation in a fungal rich background. Additionally we explore the hypothesis that mushroom mimicry could be driven by novel niche exploitation in the face of density-dependent pollinator limitation. We present several different experiments here, with the results for each immediately following the methods.

METHODS & RESULTS

Species studied

The genus *Dracula* is part of the diverse Neotropical subtribe Pleurothallidinae (Epidendroideae) which accounts for 15-20% of the species diversity in the Orchidaceae and which as a group is generally thought to be fly-pollinated, despite a paucity of empirical documentation (Van der Pijl and Dodson 1966, Pridgeon et al. 2001, Van der Cingel 2001). *Dracula* orchids (~125 spp.) are epiphytes in mature forests from southern Mexico to Peru, reaching their peak diversity in the wet forests that cover the slopes of the western Andes in Colombia (>60 spp.) and Ecuador (>45 spp.) (Luer 1993, Jorgensen and León-Yanez 1999).

Our study focuses on two species, *D. felix* (Luer) Luer and *D. lafleurii* Luer & Dalström (Figure 2.1), which represent the extremes of floral morphology within the genus. The small (8-9 mm sepals), cup-shaped flowers of *D. felix* (Figure 2.1b) with a shallowly concave labellum (4.5x2 mm) and tiny petals (3x1.25 mm) are presented singly

on individually peduncles (Luer 1993). Numerous flowers (up to 50) are produced simultaneously on each plant, with flowering concentrated in the wet season (Dec-Mar). Populations of *Dracula felix* tend to occur on the ridge tops (~1,650 m) where they can be locally abundant (data not shown). In contrast, *D. lafleurii* produces pendant, umbrella-like flowers (Figure 2.1a) with subglobose labella on descending, few-flowered racemes (~1-5 racemes at a time). *Dracula lafleurii* sepals are much larger (25-30 mm) than those of *D. felix*, as are the petals (3x2 mm) and labellum (11.5x9 mm) (Luer 1993). Plants of *D. lafleurii* continue to flower throughout the year. Our work with *D. lafleurii* was done at its type locality on the banks of the Los Cedros river at ~1,300m elevation (Luer 1993). Both species were assayed by Endara et al. (2010) for nectar production, with no detectable levels observed.

Figure 2.1. *Dracula lafleurii*, *D. felix*, and a pollinium-carrying *Zygothrica* on a mushroom. **a)** *D. lafleurii* flowers have sepals that measure 25-30 mm in length and are displayed on a successively few-flowered raceme. ©T. Policha. **b)** *D. felix* flowers measure 8-9 mm and are displayed in a profusion of single flowers borne on individual peduncles. Here a pollinium is shown being removed by a drosophilid. ©Adrian Troya. **c)** *Zygothrica* sp. standing on a mushroom (*Polyporus craterellus*) carrying orchid pollinia on its thorax ©T. Policha. (Scale bars = 1 cm.)

Study site

Los Cedros Biological Reserve (00°18'31.0"N, 78°46'44.6"W) is located between 1,250 and 2,200 m on the western slope of the Andes in northwestern Ecuador. This private reserve protects 6,900 hectares of montane cloud forest, 5,800 hectares of which is primary. The reserve is a buffer zone for the 300,000 hectare Cotocachi-Cayapas Ecological Reserve, and is part of the Chocó phytogeographical zone, recognized as one of the most biologically diverse habitats on earth (Myers et al. 2000). The reserve experiences an average annual rainfall of 2,903 mm (SE=186.41) (Jose DeCoux pers. com.). Our studies were conducted in 2008, 2010, and 2011, during the local rainy season (January-March) when the *Dracula* orchids were in peak bloom.

What are the background visitation and fruit-set rates?

Methods. - We set out to test whether there is a fitness benefit of mushroom mimicry to *Dracula* orchids compared with closely related non-mimetic sympatric species (i.e. either *Masdevallia nidifica* Rchb.f., or *M. ximenae* Luer & Hirtz depending on availability). To gauge the background rate of visitation to *Dracula* and co-occurring orchids we analyzed the control data from multiple experiments that were performed during 2008, 2010, and 2011. For each experiment we measured visits to an unmanipulated flower as a positive control; here we have combined these data to compare background visitation rates amongst *D. felix* (n=21.5hrs), *D. lafleurii* (n=10.5hrs), and *M. nidifica* (n=14.5hrs).

To determine fruit set, unopened buds were marked with a piece of green twine tied around the pedicel (or peduncle). Plants were revisited to determine fruit set. Sample sizes were: *D. felix* (n=44 buds), *D. lafleurii* (n=35 buds), and *M. ximenae* (n=23 buds).

Visitation data were summed for each 30-minute observation period, converted to an hourly rate and analyzed by a one-way ANOVA with species as the independent variable. For the fruit set data each flower was treated as an independent event and the ANOVA again used species as the independent variable. All analyses were performed in JMP® Pro 9.0.0 (SAS Institute Inc. 2010).

Results. - Visitation rates and fruit set differed among the species (Figure 2.2). In general the *Masdevallia* spp. experienced lower visitation and set less fruit than the *Dracula* spp. Visitation was highest to *D. lafleurii*, significantly higher than to the *Masdevallia* sp. ($F_{2,92}$=4.61; p=0.0124). *Dracula felix* had a significantly higher fruit set than either the *Masdevallia* or *D. lafleurii* ($F_{2,101}$=14.06; p<0.0001).

Does Dracula felix *require pollinators?*

For the unique morphology of *Dracula* orchids to be considered adaptive in terms of mimicry theory it is important to demonstrate that the resemblance to mushrooms has a fitness benefit (Roy and Widmer 1999). Floral displays represent an energetic cost, so it is assumed that they provide some fitness advantage (Chaplin and Walker 1982). Because many plants are capable of engaging in mixed mating systems, i.e. both outcrossing and self-fertilization, increased insect visitation does not necessarily mean an increase in fecundity. It is therefore critical to investigate the potential for autogamous self-fertilization before assigning importance to insect attraction.

Methods. - Twenty plants of *D. felix* were enclosed in insect-proof nylon netting (0.4mm x 0.6mm; BioQuip Products, Rancho Dominguez, CA) while they were in bud (January 5-6, 2011). The number of buds per plant ranged from two to 21. We returned 11-17 days later, and applied as many as possible of the four pollination treatments

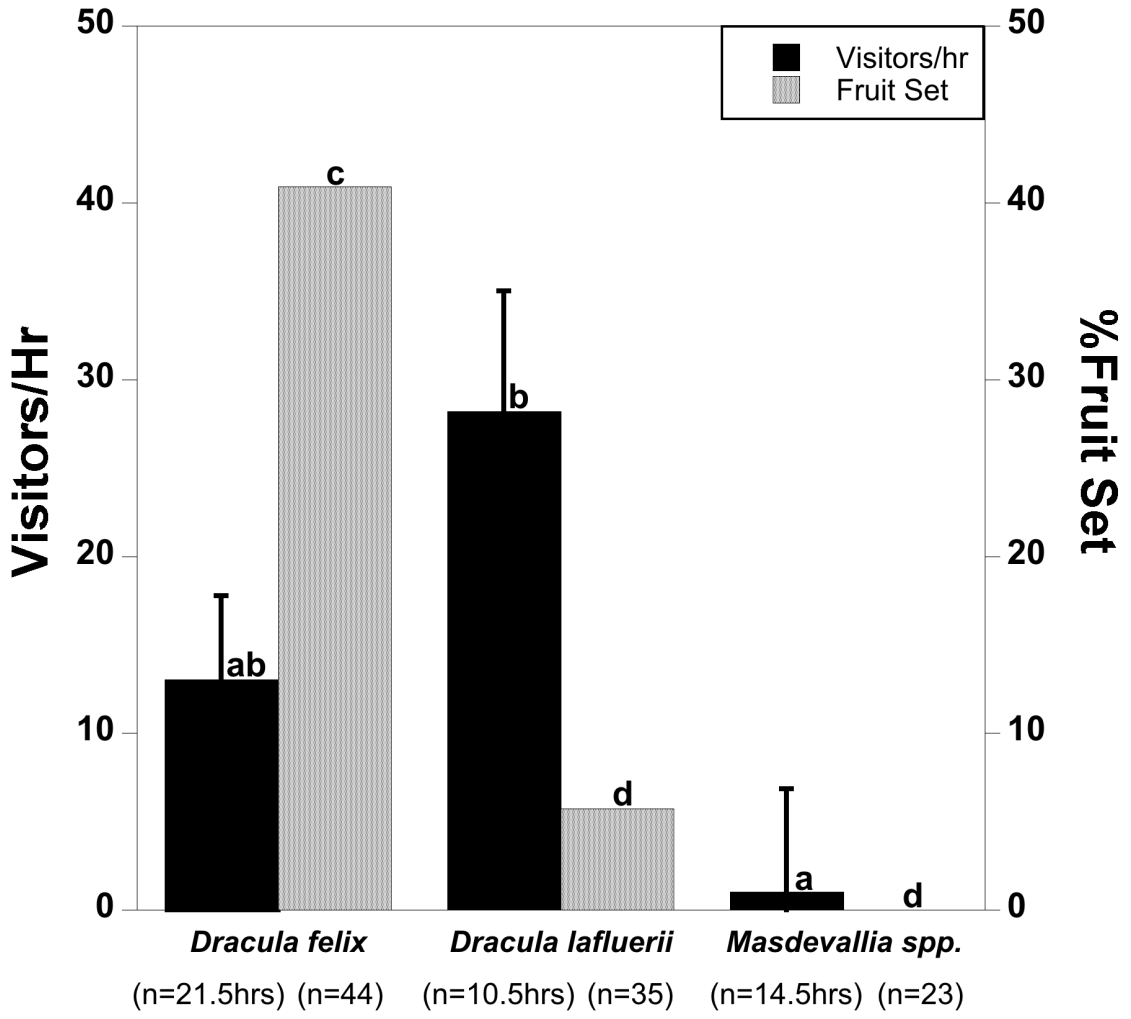

Figure 2.2. Background visitation and fruit set rates. Visitation rates and fruit set differed among the species. In general the *Dracula* spp. showed higher visitation rates and set more fruit than the *Masdevallia* spp.. *D. lafleurii* had the most visitors, significantly more than the *Masdevallia* sp. (ANOVA $F_{2,92}$=4.61; p=0.0124), and a direct contrast between *D. felix* and the *Masdevallia* sp. showed a trend towards greater visitation to *D. felix* (p=0.1136). *D. felix* fruits at a significantly higher rate than either the *Masdevallia* or *D. lafleurii* (ANOVA $F_{2,101}$=14.06; p<0.0001).

below, depending on the number of flowers available (total flowers n=102). The treatments were: 1. 'Autogamy' – where the flower was labeled, but otherwise unmanipulated; 2. 'Crossed' – where the flower was hand-pollinated with pollinia from a flower on a different plant; 3. 'Geitonogamy' – where the pollinia came from a different flower on the same plant; and 4. 'Selfed' – where pollinia came from the same flower,

but were removed and then re-inserted. Pollinations were performed with a fresh toothpick, aided by the use of a 2x optical visor (Donegan Optical Company, Inc. Lenexa, KS). After pollination the plants were re-enclosed in the mesh bags. We returned 19-34 days later to record fruit set. Not all treatments were realized on all of the plants (due to a limited supply of buds; four plants had only the 'Autogamy' and 'Crossed' treatments; and three plants received all but the 'Geitonogamy' treatment), and five of the plants received each treatment on two flowers in an effort to increase our sample size. We used a G-test (BIOMstat 3.3, Exeter Software, Setauket, NY. 1999) to determine whether the fruit set by each flower was independent of treatment or not.

Results. - Overall fruit set was lower than the background rate under natural conditions (~10% vs. ~44%). There were no significant differences in fruit set between the 'Crossed', 'Geitonogamy', and 'Selfed' flowers ('non-significant subset' G = 0.55), but each treatment produced significantly more fruit than the unmanipulated 'Autogamy' flowers, which did not set any fruits [G-test = 7.83, df = 3; p=0.0498; (Figure 2.3)]. While the number of realized fertilizations was too low to discriminate between pollen sources, it is clear that autogamy is not a viable option and that animal-mediated pollinia transfer is required for fruit set in *D. felix*.

Is visitation to **Dracula felix** *density dependent?*

Many orchids are known to be pollination limited. Nectar-less, tropical orchids in particular tend to experience low visitation rates (Tremblay et al. 2005). We predict that visitation to *D. felix* is dependent on the density of its floral display and that by masquerading as mushrooms they can apparently become part of a sufficiently abundant resource that is attractive to flies. If visitation is dependent on the density of the flowers,

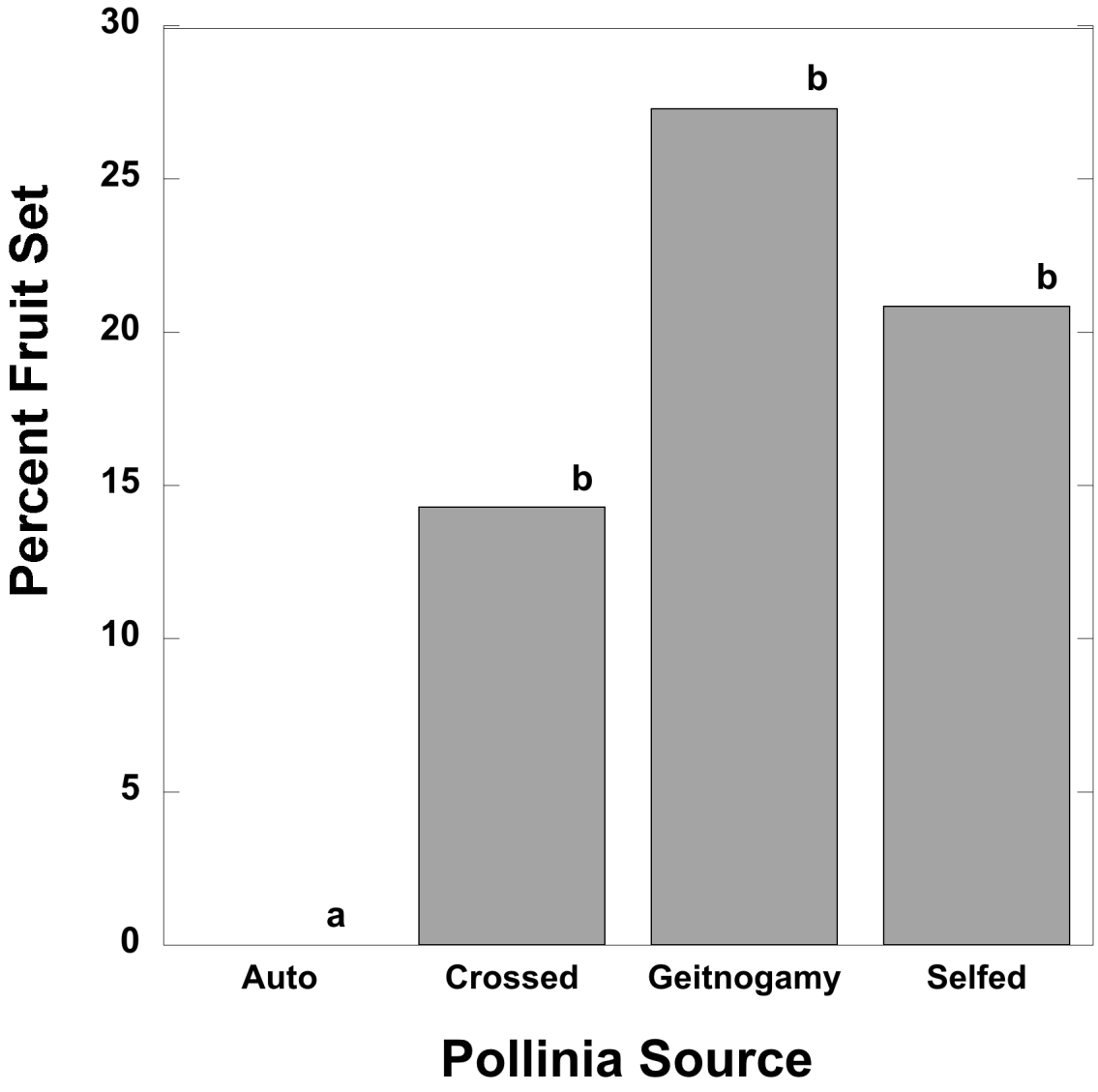

Figure 2.3. Floral visitors are required for fruit set.
A hand-pollination experiment demonstrated that animal-mediated pollination is required for fruit set in *Dracula felix*. The 'Autogamous' treatment produced no fruits and was significantly different from the other treatments (G-test = 7.83, df = 3; p=0.0498; 'Crossed,' Geitnogamy,' and 'Selfed' form a non-significant subset G = 0.55).

this would support the hypothesis that *Dracula* spp. are pollination limited, implicating this as an evolutionary driver for the fungal motif.

***Methods.* -** From January 12- 29, 2011 we counted the number of open flowers and the number of visiting flies to individuals of *D. felix* that we opportunistically

sampled along the trails. To determine the influence of floral display on the attraction of visitors, we regressed the number of flies against the number of flowers per plant. Because some plants had been counted on more than one occasion, we randomly selected one data point for each plant to include in the dataset and repeated the analysis. This controlled for the possibility that some plants would be more or less attractive for other unmeasured reasons. Regression analyses were done in JMP® (SAS 2010).

Results. - There is a strong influence of flower number on attraction of flies. In the analysis in which we used only one randomly selected data point for each of the 34 plants the r^2=0.63 and p<0.0001 ($F=55.55_{1, 32}$, Flies = -0.58 + 0.40 Flowers).

Does proximity to mushrooms influence visitation?

For resemblance to be adaptive and fit a rigorous definition of mimicry it must confer some fitness benefit to the mimicking organism (Roy and Widmer 1999). We use insect visitation as a proxy for reproductive potential, assuming that the more visitors a flower receives, the more likely for pollinia transfer to occur. Ideally, fitness would be estimated from a direct measure of seed production and viability, but this was impractical. These orchids are long-lived perennials capable of producing millions of seeds that are difficult to germinate under field conditions.

Methods: Moving* Dracula felix *flowers. - To determine the influence of proximity to mushrooms on visitation rates to *D. felix* flowers, fresh flowers were picked and displayed in small vials fabricated from 1 ml pipette tips. The flowers were rotated through three different treatments where they were observed for 30-minute periods. The three treatments were: 'Flowers' - next to (1-10cm) other flowers, 'Mixture' - next to mushrooms (1-10cm), or 'Alone' which were away from any other flowers or mushrooms

(>5m). The order in which each flower was exposed to the treatments was rotated so that there was no order bias. Each rotation through all of the treatments was considered a replicate and each flower was used for just one replicate. A visit was counted to the flower only if an insect entered the flower. These experiments were performed during January and February of 2011.

Visitation during each 30-minute observation period was translated into an hourly rate prior to analysis. Analysis was done with a one-way ANOVA where we compared the number of visits across the three treatments using JMP® Pro 9.0.0 (SAS 2010).

Results. - Overall, we observed 411 visits by drosophilid flies (25.7/hr.) and found that visitation rates differed among treatments (n=16 replicates; $F=4.29_{2, 43}$, p=0.0199; Figure 2.4a). *Dracula felix* flowers located next to other flowers received more visits than did 'alone' flowers. However, the visitation rate to flowers located next to mushrooms was not significantly different from either the flowers next to flowers or the singleton treatment, but intermediate between the two.

Methods: Moving mushrooms. - In another assay of the influence of proximity to mushrooms on visitation to *D. felix*, we performed a mushroom augmentation experiment. Instead of moving flowers next to or away from mushrooms, we moved mushrooms closer to flowers. Five stations were observed along a downed log (5m long) on which several *D. felix* plants were epiphytic. At one station we laid the 'Mushrooms' treatment, which consisted of three small, whitish bracket fungi sporocarps (*Rigidiporus* sp. Murrill; voucher #RLC-67, deposited at the Herbario Nacional del Ecuador and Royal Botanic Gardens, Kew) within two cm of each other, at three stations we laid out the 'Mixture' treatment which included three sporocarps adjacent (within two cm) to flowers,

and at a fifth station there was the 'Flowers' treatment, three individual flowers. We observed the treatments for ~3 hours over a total of 6 periods (statistical replicates). Observers took a sort break and rotated positions after each 25-minute observation period to avoid observer bias. This experiment was performed over two days to ensure independence of the replicates. All insect observations took place during January 2008 between 0900 and 1130 in the absence of rain. Visits to both flowers and mushrooms were recorded.

Visitation was translated into an hourly rate prior to analysis. We analyzed these data using ANCOVA, with the number of flowers at each station as the covariate since we know that visitation can be context-dependent (Hersch and Roy 2007). The number of flowers along the log varied among stations [from zero (in the mushroom only treatment) and then 6, 14, 47 and 65 at each of the other stations] and overall there were more flowers than mushrooms (10:1) on the log. Observations were only done on three of the flowers at each station, regardless of how many were present, and at the three mushrooms at that station. The statistical model contained the treatment ('Flowers', 'Mixture', 'Mushrooms') with the background flower number as a covariate. Analyses were done in JMP® (SAS 2010).

Results. **-** Overall, we observed 54 drosophilid visitors (5.3/hr.). In support of the mushroom mimicry hypothesis, we observed the same individual insects moving between mushrooms and flowers in the mixtures [four mushroom-to-flower transitions (7.3% of all visitors) and three from flowers to mushrooms (5.5% of all visitors)].

There was also a significant treatment effect on fly visitation rate ($F=8.66_{3, 29}$; $p= 0.0004$, whole model: $p= 0.0135$ treatment). Overall the mixtures received the most visits

while there was no difference between visits to the flowers or the mushrooms alone (Figure 2.4b). To understand these data, it is necessary to decompose the visitation rates to flowers and mushrooms. In the mixture there were 0.77±0.14 (mean±S.E.) visits per flower, whereas with just conspecifics they had 0.33±0.11 visits per flower. The fungi had 0.61±0.13 visits per sporocarp in the mixture and 0.33±0.18 per sporocarp when with conspecifics. These data show that the flowers receive more visits in mixtures with fungi than they do when just with conspecifics and that "flower" density can be augmented either with proximity to mushrooms or supplementary flowers. Additionally, in further support of the density-dependent hypothesis, the background number of flowers showed a positive slope and was significant at p= 0.0039.

***Methods: Natural and experimental variation in context and visitation to* Dracula lafleurii. -** Because each *D. lafleurii* plant typically only produces one flower at a time, we used a different approach than that used for *D. felix* to determine the influence of mushrooms on visitation. First, we looked for natural variation in the proximity of mushrooms, noting visitation rates to flowers that were very near to mushrooms (<0.5m) and flowers that were not near (>5m) to mushrooms. Flowers were monitored for 30 minutes each, with four flowers found blooming close to mushrooms and six flowers found blooming away from mushrooms.

The second test of proximity on visitation involved the experimental manipulation of flower location. Blooming plants were attached to short (about 30cm) portable sticks and located either next to (10cm), or away from (>10m) a patch of fruiting mushrooms (*Polyporus craterellus* Berk. & Curtis; voucher #RLC-717, deposited at the Herbario Nacional del Ecuador and Royal Botanic Gardens, Kew). Visitation to each of six flowers

was monitored under both treatments for 30-minute periods each and the order of treatments was alternated for each plant to avoid bias. These experiments were conducted during February and March of 2010.

For both experiments, visitation during each 30-minute observation period was translated into an hourly rate and analysis was done with a one-way ANOVA using JMP® Pro 9.0.0 in which the independent variable was treatment, and the dependent variable was visits per hour.

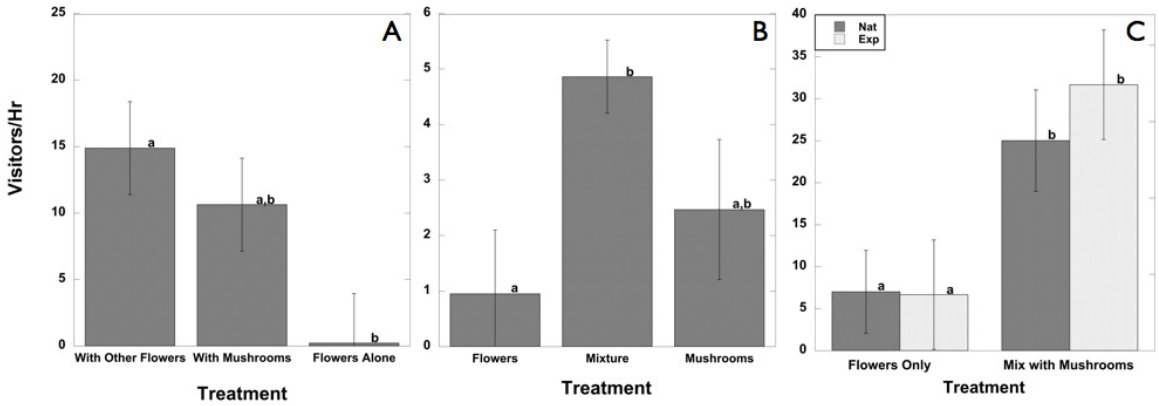

Figure 2.4. Visitation is context dependent. A. Moving flowers (*Dracula felix*). *D. felix* flowers next to other *D. felix* flowers (left-hand bar) received more visits than singleton flowers (right-hand bar). Visitation rate to flowers next to mushrooms (middle bar) was not significantly different from either other treatment. (n=16 30 min observation periods; $F=4.29_{2, 43}$, p=0.0199). **B. Moving Mushrooms (*Dracula felix*).** We observed the same individual insects moving between mushrooms and flowers: four mushroom-to-flower transitions (7.3% of all visitors) and three from flower-to-mushroom transitions (5.5% of all visitors). There was also a significant treatment effect on fly visitation rate (ANCOVA $F=8.66_{3, 29}$; p= 0.0004, whole model: p= 0.0135 treatment) with the most visits going to the mixture. The covariate, number of flowers, showed a positive slope and was significant at p= 0.0039). (n=25 25-minute observation periods). **C. *Dracula lafleurii*.** We observed higher visitation rates to flowers that occur next to mushrooms than to flowers away from mushrooms in both the experimental manipulation (black bars) (ANOVA Exp; $F=7.37_{1, 10}$; P=0.022) and the survey of natural variation (grey bars) (ANOVA Nat; $F=5.32_{1, 8}$; p=0.05). Error bars represent one standard error.

***Results.* -** We observed 71 drosophilid visitors in our natural arrays (14.2/hr.), and 115 visitors to our experimental arrays (19.2/hr.). Our results (Figure 2.4c) show significantly higher visitation rates to flowers next to mushrooms than to flowers away

from mushrooms in both the survey of natural variation ($F=5.32_{1, 8}$; $p=0.05$), and the experimental manipulation ($F=7.37_{1,10}$; $P=0.022$).

DISCUSSION

In this paper we have addressed each of the requisites for establishing mimicry laid out by Roy and Widmer (1999), and our findings support the mimicry hypothesis. Not only do these flowers exploit mushroom-visiting flies, but also their reproduction depends on it.

Overlapping distribution and phenology

We documented, with vouchered specimens, strongly overlapping distributions and phenology with co-occurring mushrooms. For example, during the peak bloom of *D. felix* we documented an average abundance of 1.82±0.40 (mean±S.E.) sporocarps/m^2 in the immediate vicinity, and 2.27±0.46 sporocarps/m^2 near where *D. lafleurii* grows (Dentinger, unpub.). The orchids and their fungal models have also co-occurred long enough for evolution to occur. The orchid lineage leading to *Dracula* and *Masdevallia* arose approximately 25 mya (Gustafsson et al 2010) and based on fossil evidence the fungi that they mimic have existed in the same habitats for at least 90 mya [Agaricales; (Hibbett et al. 1997)] or 125 mya [Agaricomycetes; (Smith et al. 2004)], providing ample time for natural selection to shape *Dracula* flowers (Gustafsson et al. 2010).

Require pollinators

Hand pollinations of *D. felix* support the necessity of insect pollination for this species. *Dracula felix* had a significantly higher background fruit set than the other species ($p<0.0001$) and the specific epithet '*felix'* comes from the Latin for '*fruitful'* and

refers to the abundant capsules produced in this species (Luer 1993). In addition to ruling out autogamy as a potential mating system, our hand pollination study shows that *D. felix* is capable of insect mediated self-pollination. This may be an evolutionary response to the fact that flies do not typically travel long distances between landings (Willmer 2011). Although we were unable to perform the same experiment on any other *Dracula* species due to low numbers of available flowers, autogamy is an uncommon mating system within the Orchidaceae compared with the elaborate strategies derived to promote outcrossing (Darwin 1862, Tremblay et al. 2005). Generally, even when selfing is possible it has been shown to have negative fitness consequences in the form of reduced growth, survivorship, or fecundity (Ellstrand and Antonovics 1985). Population genetics studies would be required to elucidate the level of genetic diversity among and between populations, and may shed light on the degree of realized out-crossing rates in other *Dracula* species (Cozzolino and Widmer 2005).

By showing that insect visitation and fruit set is higher in *Dracula* species than in co-occurring, closely-related taxa (*Masdevallia* species) that do not exhibit mushroom mimicry (Figure 2.2), we have supported the case for mushroom mimicry being adaptive in this particular environment.

Low fruit set is common in orchids, which are often limited by both pollination and resource availability, typically with the former being more important in the short term and the latter becoming significant across the reproductive life-span of the plants (Montalvo and Ackerman 1987, Primack and Hall 1990, Calvo 1993, Tremblay et al. 2005). One hallmark of hyper-diverse Neotropical cloud forests is that virtually all plant species are functionally rare (Wright 2002). This apparent rarity makes it difficult for

plants to be found in populations that are sufficiently dense to successfully attract pollinators, and is part of the basis of our hypothesis that it was adaptive for *Dracula* to expand into a novel pollination niche in the face of limited visitation by anthophilous insects. In *D. felix,* we showed that visitation is dependent on the density of the floral display ($r^2 = 0.45$; $p<0.0001$). Density-dependence is further supported by data from the experiments in which we moved mushrooms next to flowers; in that experiment, we found a significant covariate of flower number ($p=0.0039$). More flowers in the background yielded more visits.

Individuals move between the organisms

We documented 12.8% of visitors moving directly between orchids and mushrooms. We have also documented pollinia-carrying drosophilids on mushrooms (e.g., *Zygothrica* cf. *vitifrons* on *Polyporus craterellus* #RLC 717 see Figure 2.1c. and cf. *Hirtodrosophila* sp. on *Hohenbuehelia* sp. #RLC 122). Because the pollinators travel between different organisms before actually pollinating the flower, pollinia loss may be a concern. Some authors (Johnson and Edwards 2000, Harder and Johnson 2008) suggest that pollen loss during transport is a potential driver for the evolution of aggregated pollen (pollinia in the Epidendroideae). The ability within the Orchidaceae to adhere their pollinia to insect visitors also ensures conspecific pollen delivery (Johnson and Edwards 2000, Harder and Johnson 2008). Given the widespread mimicry within the orchid family (Cozzolino and Widmer 2005, Jersáková et al. 2006), it is reasonable to expect selection for pollen transfer methods that would survive transit among non-conspecific organisms. *Dracula* pollinia must survive, intact, on the bodies of flies while they travel to mushrooms (5.5% of visitors in one experiment) and then back again to a flower.

Similarity is important for fitness

Our experiments revealed that visitation rates of flies to *D. felix* flowers increased when plants were moved from isolation to close proximity with mushrooms [*a priori* contrast $F=4.15_{1,43}$, p=0.0478; (Figure 2.4a)]. There was also no significant difference in the number of visits to flowers located next to flowers compared to those located next to mushrooms, suggesting that, functionally, the flies may not distinguish between these flowers and mushrooms. In the experiment where we added mushrooms to patches of flowers, we found more visits to the floral/fungal mixtures than to either type on its own (Figure 2.4b). The fact that the contributions to this pattern came from both increased visits to flowers and increased visits to mushrooms could indicate some level of facilitation between the organisms.

With *D. lafleurii* we find a similar pattern of increased visitation in the presence of mushrooms. In this species, we focused on visits to just the flower, in part due to the difficulty keeping track of the shear volume of flies displaying on the nearby patch of mushrooms. We found that the pattern we observed under natural conditions (more visits to flowers near mushrooms) was indistinguishable from what we saw when we experimentally manipulated the system.

Overall our results support the hypothesis that *Dracula* orchids exploit the perceptual bias of their fly pollinators through adopting a "fungal phenotype." By taking advantage of a novel resource they are able to overcome the density-dependent pollinator-limitation common in epiphytic orchids. It is important to note here that these two species of *Dracula* represent opposite ends of the phenotypic spectrum within the genus. *Dracula lafleurii* is somewhat large, with a characteristically agaricoid labellum

that is made more obvious due to the open position of the sepals; *D. felix* is not only smaller, but its cup-like sepals obscure the tiny labellum from a distance (Fig, 2.1a,b). This morphological difference, combined with the profusion of flowers put forth, has led us to speculate that in *D. felix* the sepals themselves may appear as a troop of pale-capped mushrooms, which are abundant in the area (Dentinger et al. unpub.). It should also be noted that the mushrooms used in these experiments also represent a range of sizes, colors and hymenophore types. Not all of the mushrooms were agaricoids, however we have documented pollinium-carrying flies on non-gilled fungi and suspect that this is a generalized mimicry (Dentinger et al., unpub.).

Why should flowers look and smell like mushrooms? Our primary hypothesis is that the exploitation of the fungal phenotype enabled *Dracula* orchids to colonize a novel niche where competition for pollinators was low. Many orchids experience density-dependent visitation by their pollinators, leading to low levels of fruit set amongst rare or patchy taxa (Cozzolino and Widmer 2005, Tremblay et al. 2005, Brys et al. 2008). In tropical forests, due to the high plant diversity, all species are functionally rare (Wright 2002), making it difficult for plants to be found in populations that successfully attract pollinators. Low pollination success has been particularly well documented in rewardless species of orchids (Tremblay et al. 2005). Exploitation of an untapped guild of pollinators could be an effective adaptation in this veritable 'entangled bank' of biotic interactions; selection towards a mushroom phenotype would be favored in habitats in which fungi are constantly abundant. The cloud forests occupied by *Dracula* spp. support consistent levels of fungal abundance, in part due to their constantly high humidity (Talley et al. 2002) [we recorded an average of 99.8% (± 0.01 S.E.) humidity between Feb. 2010-Mar.

2012 at our study site (HOBO® Data Loggers, Onset Computer Corp. Cape Cod, MA)]. We also found that the most abundant flying insects at our field site are fungal-associated dipterans, representing a potential pollinator resource (Policha et al. unpub.)

Implications

We have established an adaptive significance for mushroom mimicry by *Dracula* spp. in the context of a Neotropical cloud forest. Despite interminable speculation that this is a Batesian system wherein the flies are duped into doing something that does not promote their own fitness, our data suggest that we may need to rethink how this enigmatic example of natural selection fits into what we know about mimicry theory more broadly. It is still unclear exactly what resources are being sought by the pollinators of *Dracula*, both at the flowers and the fungi. We have observed courtship behaviors and mating on both, the mushrooms and orchid flowers, as well as a lapping behavior that is indicative of either tasting chemicals or feeding on superficial microbes. Endara et al. (2010) reported similar lekking-type behaviors in their documentation of the pollination of these *Dracula* species, and similar courtship displays performed on mushrooms have also been described by Grimaldi (1986) and Burla (1990) for other species of *Zygothrica.* An outstanding question is whether or not the flies lay eggs in the mushrooms or the flowers where they congregate and mate. Flies in the genus *Zygothrica* typically court and mate on mushrooms, but then oviposit in flowers, with only 10% of the described species ovipositing in mushrooms (Courtney et al. 1990). Flies in the genus *Hirtodrosophilia* on the other hand are mostly mycophagous in the larval stage (Courtney et al. 1990). Most of the visitors to *D. felix* that we observed appear to belong to *Zygothrica, Hirtodrosophila*, or the closely related *Laccodrosophila* (Grimaldi pers.

com.). If the pollinating flies use mushrooms for courtship but lay their eggs in flowers, increased visitation in a mixture could represent a case of resource partitioning, as opposed to deception. Alternatively, if the flies also successfully utilize the mushrooms as brood sites, then the mixture may represent a larger overall resource. If future experiments on resource use find that the proportion of larvae hatching from both flowers and mushrooms are the same, then it would not be appropriate to refer to this system as Batesian mimicry, since the flies would be receiving the same benefit from both model and mimic.

While studying host selection by mycophagous *Drosophila* spp., Jaenike (1978) found that there was little specificity in substrate selection for courting, mating, and ovipositing sites amongst the species studied and suggests that this lack of preference could reflect an acclimatization to the ephemeral resource of fungal fruiting bodies. This potential background lack of constancy could represent a more Müllerian-like convergence (between *Dracula* spp. and mushrooms) that would facilitate host switching by these flies. This in turn could provide selection for the evolution of the mushroom phenotype in *Dracula*. Future papers will explore the exploitation of a perceptual bias in the flies in the evolutionary history of mimicry within *Dracula*. Ongoing surveys of both the fungi and flying insects, especially the mycophagous community, will help us understand the biological links between the different species of flies and fungi, shedding light on the ecology and evolution of this enigmatic mimicry system.

BRIDGE TO CHAPTER III

In this chapter we examined how the resemblance to mushrooms may confer a fitness advantage to *Dracula* orchids occurring in a fungal rich environment. We show that some species of *Dracula* elicit higher rates of visitation than their non-mushroom mimicking relatives (e.g., *Masdevallia nidifica*). We also show that some species of *Dracula* exhibit higher rates of fruit set than their non-mushroom mimicking relatives at our study site. Using a hand-pollination experiment we demonstrate that insect visitation is critical for effective pollination and subsequent fruit set in *Dracula felix*. The presence of mushrooms increases visitation rates to the orchids. We determined this by relocating individual flowers and whole plants into different contexts, both away from and into proximity to mushrooms. We found a similar pattern when we repositioned mushrooms next to *Dracula* flowers. These data taken together with the visitor identifications in chapter IV suggest a fitness benefit to *Dracula* orchids by exploiting a dipteran fauna that otherwise spend time on and around mushrooms. By establishing that insect visitation is indeed important for the reproduction of these plants, and that they recieved more landings in proximity to mushrooms, the next investigation in our study is an exploration of the mechanisms whereby *Dracula* flowers attract these insects. In chapter III we focus on *Dracula lafleurii*. Using traditional bagging techniques, novel, 3D-printed, artificial flowers, and chemical analysis of the olfactory cues we disentangle the contributions of visual and olfactory signals in the attraction of these mushroom-associated flies to *Dracula* flowers.

CHAPTER III

DISENTANGLING VISUAL AND OLFACTORY SIGNALS IN MUSHROOM-MIMICKING *DRACULA* ORCHIDS USING REALISTIC 3-D PRINTED ARTIFICIAL FLOWERS

The floral deconstruction experiment was designed by Bitty Roy, Bryn Dentinger and myself. The floral reconstruction experiment was designed by Bitty Roy, Bryn Dentinger, Melinda Barnadas, Rob Raguso and myself. The chimera experiment was designed by Bitty Roy and Aleah Davis (who collected the data). The color/contrast experiment was designed by Bitty Roy and Melinda Barnadas and carried out by Bitty Roy and Aleah Davis. Rob Raguso helped analyze the volatile chemistry and contributed to the theoretical framework of the paper. I coordinated the data collection for all experiments not otherwise noted above, did the bulk of the volatile analysis, performed all of the statistical analysis and wrote the chapter with editorial input from these co-authors.

INTRODUCTION

Floral mimicry and deceptive pollination have been recognized for over 200 years (Sprengel 1793), but the details of the perceptual biases involved in the evolution of floral mimicry remain an active field of investigation (Dafni 1984, Schiestl 2005, Jersáková et al. 2006, Vereecken and McNeil 2010). Flowers evolve in response to the perceptual biases of pollinators for traits such as scent, color and pattern (Schaefer and Ruxton 2009, Schiestl and Dötterl 2012, Papadopulos et al. 2013). Small differences in perceptual acuity and bias can be exploited by selection if it imparts an increase in fitness (Chittka et

al. 2001). Perceptual biases do not have to be innate, they can also be learned preferences, or can emerge from a combination of causes (Schaefer and Ruxton 2009). From the floral perspective, it does not matter which mechanism imparts the bias; natural selection on the flowers will favor individuals that match the perceptual biases of their most effective pollinators most closely, if these increase the fitness of the plants.

Legitimate fly pollination (myophily) is widespread across angiosperms (Larson et al. 2001), and flies are also exploited in various ways to act as pollinators in deceptive scenarios, including brood site mimicry (Burgess et al. 2004, Van der Niet et al. 2011, Jürgens et al. 2013), yeast mimicry (Goodrich et al. 2006, Stokl et al. 2010), and sexual deception (Johnson and Midgley 1997, Blanco and Barboza 2005, Ellis and Johnson 2010, Gaskett 2011). Due to their ubiquity, flies represent an important source of pollination services for plants (Larson et al. 2001, Ssymank et al. 2008). In moist, shady habitats such as tropical cloud forests, which are typically poor in anthophilous insect fauna but rich in mushroom-associated taxa, the evolution of pollination by these mushroom-associated flies should be favored (Mesler et al. 1980)..

One genus of putative mushroom mimics is *Dracula* Luer (Orchidaceae). *Dracula* orchids are curious because their labella look like co-occurring mushrooms (Vogel 1978, Dentinger and Roy 2010), they produce the same volatile compounds that give mushrooms their characteristic odors (Kaiser 1993a, Kaiser 2006), and they are pollinated by mushroom-visiting flies (Endara et al. 2010). Here we examine the signaling motifs enabling this mimicry. Is the mimicry multi-modal, utilizing visual and olfactory cues? Or is odor the primary attractant, as is typical in carrion brood-site mimicry systems (Stensmyr et al. 2002, Moré et al. 2013), with visual or other cues contributing

secondarily? In addition to the mushroom-like labellum *Dracula* flowers display rather large and showy fused sepals (Figure 3.1), suggesting that there may be a significant visual component independent of the mushroom motif.

The flying-insect community at our field site in Ecuador is dominated by dipteran groups known to include mushroom-visiting members (Phoridae, Sciaridae, Mycetophilidae) (unpublished data), and the flies that we find at the *Dracula* flowers come from largely mycophilous genera of the Drosophilidae (Endara et al. 2010 and unpublished data). Mushroom-visiting flies are often polyphagous as a consequence of utilizing the ephemeral resource of fungal fruiting-bodies (Jaenike 1978). As fungal generalists, these insects may use search images that are particularly susceptible to exploitation by mimetic interlopers. Mimics may take advantage of the range of model phenotypes to avoid learned avoidance in the visitors (Roy and Widmer 1999). Models of associative learning in mimetic context suggest that learned avoidance should take longer when fraudulent signals are more variable (Balogh et al. 2008). Our data on the mushrooms that co-occur with *Dracula* orchids indicate that there is no single model (Dentinger and Roy 2010, and unpublished data), but instead it appears to be a generalized mimicry system where there is a convergence on the mean phenotype of several potential models. In this situation, *Dracula* flowers are likely to benefit from the exploitation of perceptual biases in the floral visitors (Ruxton and Schaefer 2011).

We focus here on *Dracula lafleurii* (Luer & Dalström) (Figure3.1), which blooms for several months and has relatively large flowers. We begin by demonstrating that both visual and olfactory cues are important and then experimentally dissect each aspect in detail. To address perceptual bias questions we fabricated life-like flowers from scentless

silicone using a combination of casting and 3-D printing methods. These artificial flowers allowed us to decouple sensory aspects of the phenotype and assess their attractiveness to visitors individually. Because each part of the flower was produced separately (see Methods), the parts were interchangeable, allowing the construction of 'chimeras' from various real and artificial flower parts. By using a series of floral deconstruction, floral reconstruction, and chimera experiments we were able to tease apart the relative importance of visual and olfactory cues as well as assign roles to each flower part in this complex, multi-modal signaling strategy. *Dracula* orchids are unique in the mushroom-like morphology of the lower labellum (Figure 3.1), but they also show impressive diversity in the morphology of the remaining floral organs (Luer and Escobar 1988). For example, there is a range of sizes, shapes, colors, pubescence, and appendages in the sepals, and even within a given species there can be a high level of variation (see Appendix A). The artificial flowers we fabricated allowed a detailed investigation of the complex visual aspects of the large showy calyx decoupled from the volatile phenotype associated with the labellum.

Our results show that both visual and olfactory components play a role in attraction in this system and suggest that each floral part makes a discernable contribution to recruitment of dipteran pollinators. We show that the labellum – the feature that defines the genus *Dracula* – is largely responsible for effective mushroom mimicry, and we discuss possible functional roles for and evolutionary shifts involving the showy calyx.

METHODS

Study site

The field work described here was performed at Los Cedros Biological Reserve (type locality for *Dracula lafleurii* (Luer 1993)) which is located between 1,250 and 2,200 m elevation on the western slope of the Andes in the Imbabura Province of northwestern Ecuador (00°18'31.0"N, 78°46'44.6"W). This private reserve protects ~7,000 hectares of mostly primary montane cloud forest (Sierra 1999), abutting the 305,000 hectare Cotocachi-Cayapas Ecological Reserve, and is part of the Chocó phytogeographical zone (Guevara and Campos 2003), recognized as one of the most biologically diverse habitats on earth (Myers et al. 2000). Rainfall is high, averaging 2,903±186 mm per year, based on records kept at the reserve (J. DeCoux unpub.). Our studies were conducted during the local rainy season (January-March) 2010-12, when the *Dracula* orchids were in peak bloom. The focal populations of *D. lafleurii* were situated around 1,300 m elevation next to the Rio Los Cedros. The forest canopy here reaches to ~25m and supports abundant mosses and vascular epiphytes. Average canopy cover ranges from about 75-80%. Previous workers estimate 300 tree species per hectare based on the collection of 2,744 individual specimens of 337 tree species, from 40 genera and 61 families. The five most common families of trees are Urticaceae (18%), Lauraceae (14%), Melastomataceae (9%), Rubiaceae and Moraceae (6%) (Peck et al. 2008).

Dracula lafleurii ***(Luer & Dalström) and visitors***

The genus *Dracula* (~125 spp.) is part of the diverse Neotropical subtribe Pleurothallidinae (Epidendroideae) which accounts for 15-20% of the species diversity in the Orchidaceae, and which as a group is generally thought to be fly-pollinated (Van der

Pijl and Dodson 1966, Pridgeon et al. 2001, Van der Cingel 2001). *Dracula* orchids are epiphytes in mature forests from southern Mexico to Peru, reaching their peak diversity in the western Andean cloud forests of Colombia (>60 spp.) and Ecuador (>45 spp.) (Luer 1993, Jorgensen and León-Yanez 1999).

This study focuses on *Dracula lafleurii* (Figure3.1), which produces pendant, umbrella-like flowers with subglobose labella on descending, few-flowered racemes (~1-5 racemes at a time; Figure 3.1). The calyces range from 411-1623 mm^2 in area, the petals are 3x2 mm. in size and the labellum averages ~1cm in width. The flowers do not produce detectable levels of nectar (Endara et al. 2010).

Dracula lafleurii is visited almost exclusively by small flies (Figure 3.1) in the family Drosophilidae (Endara et al. 2010). We have collected ~20 different species on these flowers (data not shown), mostly from the genus *Zygothrica* (Drosophilidae), most of which are undescribed (Grimaldi pers. comm. and Policha etal. unpub.). These visitors display a range of behaviors on the flowers, including standing (sheltered from the rain), walking (flies are known to taste with and their feet (Dethier 1976, Barth 1985) so this may be related to the following behaviors), lapping at the surface and apparently consuming yeasts that occur there (McAlpine 2013, and unpub.). They also display courtship-associated wing movements (Grimaldi 1987, Burla 1990), and territorial behavior; mating is also (rarely) observed. Visitors spend between seven seconds to over 30 min. within and upon the flowers. Pollinia removal is often realized after an individual wedges itself deep within the flower, spending several minutes beneath the column. In order to reach the column, the fly must cross the inner labellum of the flower.

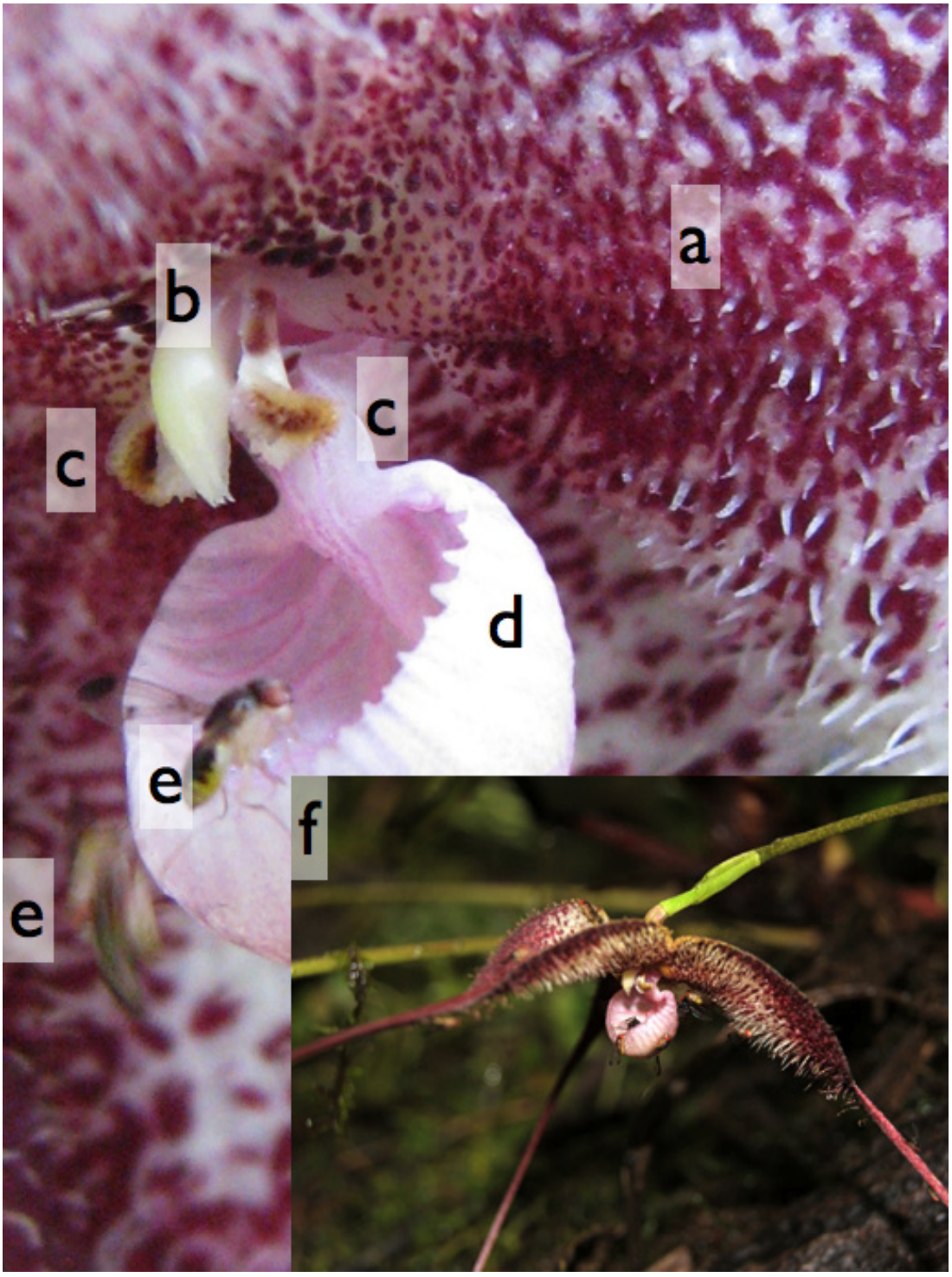

Figure 3.1. ***Dracula lafluerii*****.** Produces pendant, umbrella-like flowers with subglobose mushroom-like labella on descending, few-flowered racemes. The type locality is our research site at Reserva Los Cedros. **a)** calyx; **b)** column (pollinia attached on the underside); **c)** lateral petals, **d)** labellum with radiating 'gill-like' ridges (~1cm across for scale); **e)** floral visitors in the genus *Zygothrica*; **f)** Inset shows whole flower (with more drosophilid visitors). Photos © B. A. Roy.

Artificial flowers

Using 3D printing technology, we manufactured artificial flowers (Figure 3.2) using odor-free, pharmaceutical-grade silicone that could be scaled to specific proportions. Molds were created by dissecting flower parts and casting them in a dental-grade aqueous elastic impression material [a mix of kelp-derived potassium alginate and calcium sulfate (www.renewmaterials.com)]. These temporary alginate molds were used to create more stable positive casts using a very fine, high strength plaster [Hydrocal FGR-95 Gypsum Cement (USG Corporation, Chicago, IL)]. The plaster positives were scanned into high-resolution 3D images at the Arius 3D Imaging Centre at the Canadian Museum of Nature, (Gatineau, QC.), and digitally adjusted to user-specified proportions. The finalized 3D images were printed as cyanoacrylate impregnated gypsum molds on the ZCorp Spectrum 510 at the Scripps Physical Model Service, of the Molecular Graphics Laboratory at The Scripps Research Institute (La Jolla, CA). The final synthetic flower parts were created using a fragrance-free, platinum-cured, pharmaceutical grade silicone [SILBIONE® RTV 4420 A/B (Bluestar Silicones USA Corp. East Brunswick, NJ)]. Pigments were encapsulated in the silicone before casting to achieve the desired coloration for each artificial flower without odors from the pigments. Color matching was based on sampling and averaging of the wavelengths of the colors in the actual flower. Live flowers were collected (Andy's Orchids, Encinitas, CA.) and color-matched using a spectrophotometer (Shimadzu UV-1650; range 190nm-1110nm; deuterium and tungsten-halogen lamps) at the Environmental Engineering Lab, Department of Civil, Construction, and Environmental Engineering at San Diego State University.

Deconstruction of olfactory and visual cues

To address the hypothesis that either visual or olfactory signals are sufficient to attract floral visitors we selectively isolated each native aspect of the signaling phenotype. Sites (n=6) with at least two open and attractive flowers were subjected to the following treatments: 1. An unmanipulated living flower (positive control); 2. A living flower enclosed within a transparent, odor-impermeable, nylon-resin oven bag (Reynolds®)(visual only); 3. An empty oven bag (visual negative control). 4. A living flower masked within a green muslin cloth bag (odor only); 5. An empty green muslin cloth bag (odor negative control). Masked treatments ('odor only' or 'visual only') were observed next to one of the unmanipulated flowers and the respective negative control (Figure 3.2a) in random order for ten 30 min. replicates (5 hrs total). All observations were made between January 31 and February 10, 2010.

Reconstruction of olfactory and visual cues

Pollinator attraction can be multi-modal with olfactory and visual cues being synergistic (Raguso and Willis 2002, Raguso and Willis 2005). To test this hypothesis in *Dracula lafleurii*, life-like artificial flowers were presented in arrays that included five treatments: 1. A living flower (positive control); 2. An artificial flower (visual only); 3. A green artificial flower (material negative control); 4. An artificial flower augmented with a volatile solvent extract (odor + visual); and 5. An artificial flower augmented with solvent only, as a (solvent negative control) (Figure 3.2b). The added volatile extracts were prepared by separately soaking the calyx, and the corolla/column of *D. lafleurii* flowers in 0.5 ml of a 9:1 hexane:acetone solvent (Kaiser 2004) for 6 hours and then diluting the supernatant with mineral oil (2:1 extract:mineral oil). Volatile extracts from

the calyx, which is the showy part of these flowers (Figure 3.1), was applied to the calyx of the artificial flower and the extract from the column and corolla (inc. labellum) of the flowers was applied to the labellum of the artificial flower. Each observation period was 30 min. long (n=18 (9 hrs total)). A total of 37 visitors were observed. For analysis the unscented Visual Only' artificial flower was compared to the green artificial flower ('Material Control') to establish the effect of visual cues alone. The artificial flower with the added volatiles ('Odor+Visual') was contrasted against the artificial flower coated with just the solvent to control for the fact that the hexane:acetone:mineral oil blend may influence attraction and visitation. All observations were made between January 15 and March 7, 2011.

Chimeras of living flowers and silicone parts

By employing chimeric flowers made of both real and artificial parts we were able to retain more of the native attractiveness of the discrete floral organs than with the scented artificial flowers. At a gross scale we predict that the large showy calyx is responsible for the bulk of the visual signals, while our preliminary chemical analyses showed that the labellum and column of *Dracula lafleurii* are responsible for almost all of the total odor bouquet and that the 'mushroomy' volatile 1-octen-3-ol is concentrated in the labella (Figure 3.8b). It is important to recognize other potentially consequential floral characteristics as well. By utilizing real flower parts, we are presenting the contextual and tactile cues of the pubescent calyces, we know that the real labella are covered in yeasts that may play a role in both attraction and retention (McAlpine 2013 and unpub.). Teasing out these additional potential sources of pollinator attraction or behavioral modification is beyond the scope of the present experiment.

Flower parts were dissected by removing the calyx from the rest of the flower by incising around the column and corolla ('labellum') with a razor blade, and then combined with the complementary artificial parts to build the chimeras (Figure 3.2c). Because the artificial flowers were designed to have interchangeable parts, the combination with the real flower parts was possible using friction to hold the flower pieces in place, thus avoiding the use of an additional adhesive material, which may have introduced confounding volatiles. We used a fully crossed design in which the four treatments included every combination of living and artificial flower parts: a Real Flower, an Artificial Flower, a Real Calyx Chimera (with an artificial labellum), and a Real Labellum Chimera (with an artificial calyx). To control for volatiles released by the flower in response to tissue damage, the calyx of the intact flower was inconspicuously cut near the joint of the calyx and labellum. Artificial and chimeric treatments were randomly arranged around the real, flowering *D. lafleurii* plants. These data were analyzed by ANOVA and post hoc (Tukey's HSD) tests. Since we were using real flower parts, we could not rule out the possibility of similar visitation rates as to the real flower, and performing all of the pairwise contrasts would violate the allowable number of independent tests. Thirteen 30-minute observation periods (6.5hrs. total) were conducted between February 8 and 15, 2011.

Visual signals: the roles of contrast, pattern and color

The calyx of Dracula lafleuri flowers is large, showy, and highly variable [Supplemental Figure S3.1 (Appendix A)]. We quantified this variation in size and coloration using field measurements and the imaging software Image J (Rasband 1997-2012) [Supplemental Figure S3.2 (Appendix A)]. The unifying motif in the *Dracula*

lafleurii calyx is a white background with red-maroon spots on it, variously arranged in linear or dispersed fashion, displaying marked contrast and patterns [Supplemental Figure S3.2 (Appendix A)]. To explicitly test the relative contributions of color, pattern and contrast in attracting visitors, we observed visitation rates to five treatments including four different artificial silicone flowers: a real flower in the field, two 50% maroon/white artificial flowers, 'spotted' and 'striped', and solid 'red' and solid 'white' artificial flowers (Figure 3.2d & Figure 3.7). In order to explicitly test the role of the various visual cues, no volatiles were applied to any of the artificial flowers in this experiment. While the calyx varied across the artificial flower treatments, the labella (and associated structures – lateral petals and column) were fabricated in white silicone and held constant across the artificial flower treatments. We put the fabrications out in a natural population of *Dracula lafleurii* along the Rio Los Cedros next to a flowering individual. Each observation period was 30 min. (n=6 (3 hrs total)). Because we did not expect any of the artificial flowers to be as attractive as the real flower, it was used as a positive control in order to determine whether *Dracula*-visiting flies were present, but it was not included in any of the analyses. All observations were made between January 22 and February 15, 2011. Visitor observations were done between 900 and 1300, under relatively dry conditions (ranging from no rain to light drizzle).

Since UV reflectance can play an important role in insect attraction (Menzel 1975, Peter and Johnson 2008) we needed to determine whether this would be an important aspect of visual signaling to include in the artificial flowers. From a limited (n=1) assay of greenhouse grown *Dracula lafleurii* flowers (Marsh Hollow Orchids, Fenwick, Ont.), what little UV reflectance there may be appears to come primarily from the column

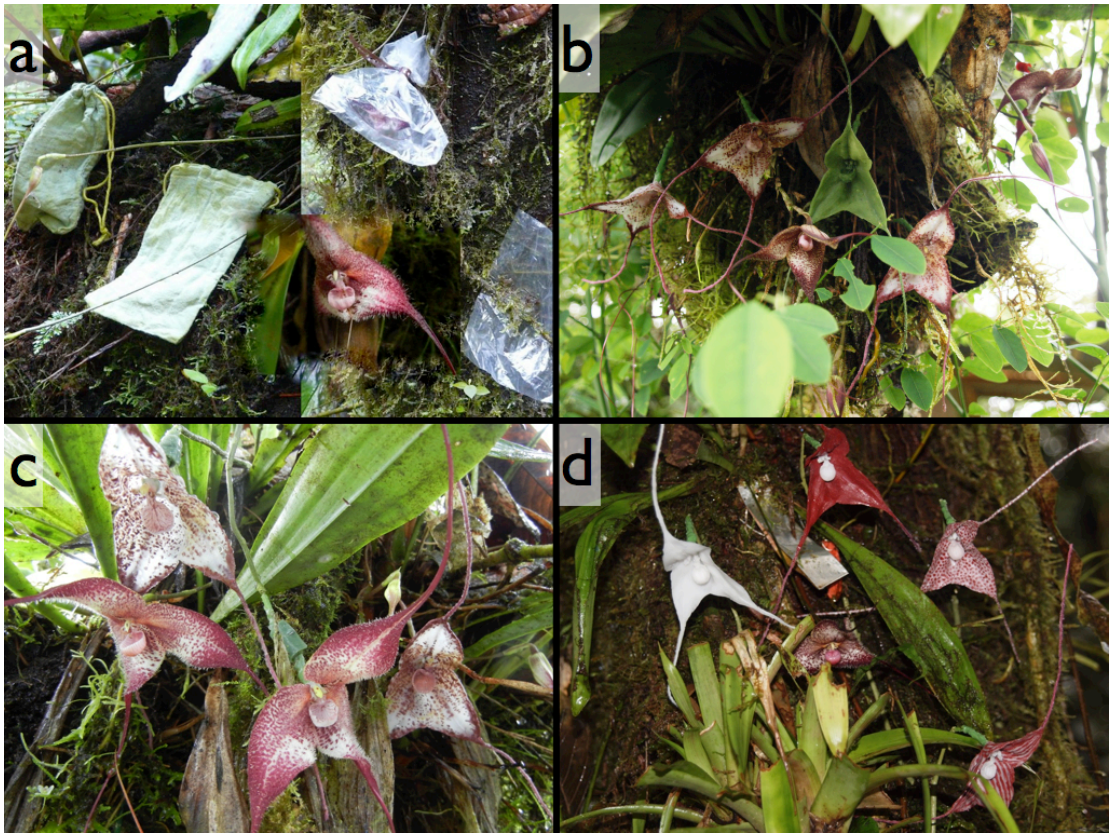

Figure 3.2. a) Deconstruction of olfactory and visual cues. To selectively isolate each aspect of the signaling phenotype, sites with at least two open flowers were subjected to the following treatments: 1. An unmanipulated living flower (positive control) (middle); 2. A living flower enclosed within a transparent, odor-impermeable, nylon-resin oven bag ('Visual Only') (right upper); 3. An empty oven bag ('Visual Control') (right lower). 4. A living flower masked within a green muslin cloth bag ('Odor Only') (left upper); 5. An empty green muslin bag ('Odor Control') (left lower). Because each site rarely had more than two open flowers at a time, the masking treatments were applied consecutively instead of at the same time. This figure is therefore a collage of photos representing the different treatments. All photos © R. Manobanda. **b) Reconstruction of olfactory and visual cues.** Life-like artificial flowers were presented in arrays that included five treatments: 1. A living flower ('positive control') (lower middle); 2. An artificial flower ('Visual Only') (upper middle); 3. A green artificial flower ('Material Control'); 4. An artificial flower augmented with a *Dracula* volatiles extracted in solvent ('Odor+Visual') (right); and 5. An artificial flower augmented with solvent only, as a ('Solvent Control') (left); The added volatile extracts were prepared by soaking *D. lafleurii* flowers in a 9:1 hexane:acetone solvent and then diluting the supernatant with mineral oil (2:1). There is an additional flower in the upper right that was not a part of the experiment. Photo © B. A. Roy. **c) Chimeras of living flowers and silicone parts.** Four treatments included every combination of living and artificial flower parts: 1. a real *D. lafleurii* flower (lower left), 2. a totally artificial flower (upper left), real sepals with an artificial labellum (RCAL) (middle) and artificial sepals with a real labellum (ACRL) (right). Photo © A. Davis. **d) Visual signals: Contrast, pattern and color.** Five treatments including four different artificial silicone flowers: a real flower in the field (middle), two 50% maroon/white artificial flowers, spotted and striped (right, upper and lower respectively) and solid red and solid white artificial flowers (upper middle and left respectively). Photo © B. A. Roy.

[Supplemental Figure S3.3 (Appendix A)]. In a separate experiment to determine if the ultra-violet component of the labellum influenced visitation in the field, we assayed visits to artificial flowers that had any of, a white, a UV-reflective, or a UV-fluorescent labella. The UV-reflective labella was fabricated by dehydrating, powdering, and adding Bird Vision UV Decoy Paint (Reel Wings Decoy Co. Inc. Fargo, ND) to the silicone before creating the labellum. The UV-fluorescent labellum used Fluorescent Pigment White No.56000 from Kremer Pigments Inc. (New York, NY) added to the silicone as a dry pigment. Fluorescent pigments are luminescent materials that require no artificial energy to reflect colored light and to give off fluorescent light (Streitel 2000).

The white labella were utilized in the visual cues experiment (above) after no difference was detected in the attractiveness of the various UV or plain white labella [Supplemental Figure S3.3 (Appendix A)].

Experimental design and statistical analysis

All experiments were set up in a 'Randomized Complete Block' design with observational period as the block. Analysis was done by a 2-way mixed-model ANOVA with block (random), treatment (fixed) and the interaction term (block x treatment) in the model. The response variables for all experiments were visitation rate (landings/hour) to each of the treatments and the duration of each visit (seconds/visit). Additionally for the first two experiments (deconstruction and reconstruction) we also analyzed approaches, which were defined as directed travel to within 5 cm. of the target. All approaches are included in this count, including both those that result in a landing on the flower, and those that do not. All analyses were done with the program JMP 9.0.1 (SAS 2010) on log-

transformed data (to deal with skewed residuals). For ease of interpretation we present untransformed data in the figures.

For the chimera experiment we also made detailed records of where the insects first landed on each treatment and how they moved within a treatment once they were there. These movements are diagramed to illustrate relative frequencies of landings and transitions (Figure 3.5). Preference in landing location was determined by using G-tests to compare our observed distributions to the null hypothesis of no preference (equal lands to each location) (Sokal and Rohlf 1995). Additionally, we combined the first-land location data across treatments to determine what floral aspect was driving this difference in preference using combined G-tests (Sokal and Rohlf 1995). These statistical test were performed in BIOMstat 3.3 (Rohlf 1999).

Volatile chemistry

To evaluate the hypothesis that *Dracula* orchids uniquely smell like mushrooms we analyzed the contents of the volatile bouquets of the *Dracula* flowers in comparison to the co-occurring mushrooms and the other co-occurring pleurothallid orchids lacking mushroom-like labella. Specifically we asked: do the *Dracula* volatiles match those of fruiting fungi present in the same habitat? Do they depart substantively from the volatile bouquets of sympatric, related pleurothallid orchids (simultaneously controlling for habitat and phylogeny)? Whole flowers, flower parts (calyx, (lateral) petals, labellum, column), and mushrooms were extracted in 500 μl of 9:1 hexane:acetone for 6 hours. Solvent controls were also collected for each time and place that extracts were made to account for ambient odors and/or contaminated glassware.

Prior to analysis all samples were filtered through quartz wool, concentrated to 50 µl under N_2 gas, and 5 µl of a 0.03% toluene solution (in hexane) was added as an internal standard. Aliquots (1 µl) of the concentrated extracts were injected (splitless) into a Shimadzu GC-17A (with Shimadzu AOC-20i autoinjector), equipped with a Shimadzu QP5000 quadrupole electron impact MS (Shimadzu Corporation. Kyoto, Japan) as a detector, on a highly-polar ethylene glycol capillary column (EC™ Wax; W. R. Grace & Co. Columbia, Maryland) (30m x 0.25 mm i.d., film thickness 0.25 µm). Sample blends were separated using one of two temperature programs; temperature program 1 (30 min.; exploratory, to screen all volatiles): inject at 40°C and hold for 3 min, then increase by 10°C/minute to 260°C and hold for 5 min.; or temperature program 2 (20 min.; after no high-boiling compounds were found, truncated to reduce time of analysis): inject at 40°C and hold for 3 min, then increase by 10°C/minute to 200°C, then increase by 30°C/minute to 260°C and hold for 9 sec. The carrier gas was ultra high purity (99.999%) helium, with a flow rate of 1.5 ml/min. (20:1 split ratio), and the column pressure at injection was maintained at 61 kPa.

Compounds were identified using computerized mass spectral libraries (Wiley, NIST and Adams), and verified using retention times and mass spectra of authentic reference standards (Raguso et al. 2006). Focusing on a suite of the eight most common compounds that were in our preliminary *Dracula* samples, we assessed similarities in scent bouquets between the pleurothallid orchids, *Dracula lafleurii,* and mushroom groups by using Non-metric Multidimensional Scaling (NMDS) with Sørenson (Bray-Curtis) distance and compared with Multi-response Permutation Procedures (MRPP) performed with PCOrd 6.14 (McCune and Mefford 2011).

RESULTS

Deconstruction: both olfactory and visual cues attract floral visitors

During ten 30 min. observation periods 420 flies were recorded approaching the array of bagged and unbagged flowers of which only 130 actually landed. Approaches to the array depended on treatment. Individual contrasts show differences between the 'masked' treatments and their negative controls; the flower bagged in plastic ('Visual Only') elicited more approaches than its control, the empty plastic bag ('Visual Control') $F_{1,33.7}=14.05$; p=0.0007 (Figure 3.3a). The flower bagged in green muslin ('Odor Only') also received more approaches than its 'Odor Control,' an empty green muslin bag; $F_{1,33.7}=4.02$; p=0.0531 (Figure 3.3a). Neither actual landing rates (Figure 3.3a) nor visit duration (data not shown) showed significant differences between the 'masked' treatments and their controls. The results presented here omit three outlying data points of landing rates to the 'Real Flower', all of which were greater than twice the standard deviation above the mean. When these data were included we saw a similar pattern, but returned different significance levels ('Visual Only' vs. 'Visual Control' $F_{1,36}=9.63$; p=0.0037; 'Odor Only' vs. 'Odor Control' $F_{1,36}=2.76$; p=0.1056).

Floral reconstruction: odor extracts added to a visual model stimulate landings

During eighteen 30 min. observation periods 97 flies were recorded approaching the array of scented and unscented artificial flowers of which only 37 actually landed (Figure 3.3b). Approaches depended on treatment. Both artificial flower treatments ('Visual Only' and 'Odor+Visual') were more attractive than their negative controls [(contrast) $F_{1,68}=4.13$; p=0.0459, and (contrast) $F_{1,68}=10.62$; p=0.0017, respectively]. However, the added volatile extracts were required to actually evoke a landing. Landing

rates to the 'Odor+Visual' treatment were marginally higher than to its negative control ($F_{1,68}$ =3.46; p=0.0671). Visit duration (data not shown) showed no significant differences between the treatments and their controls.

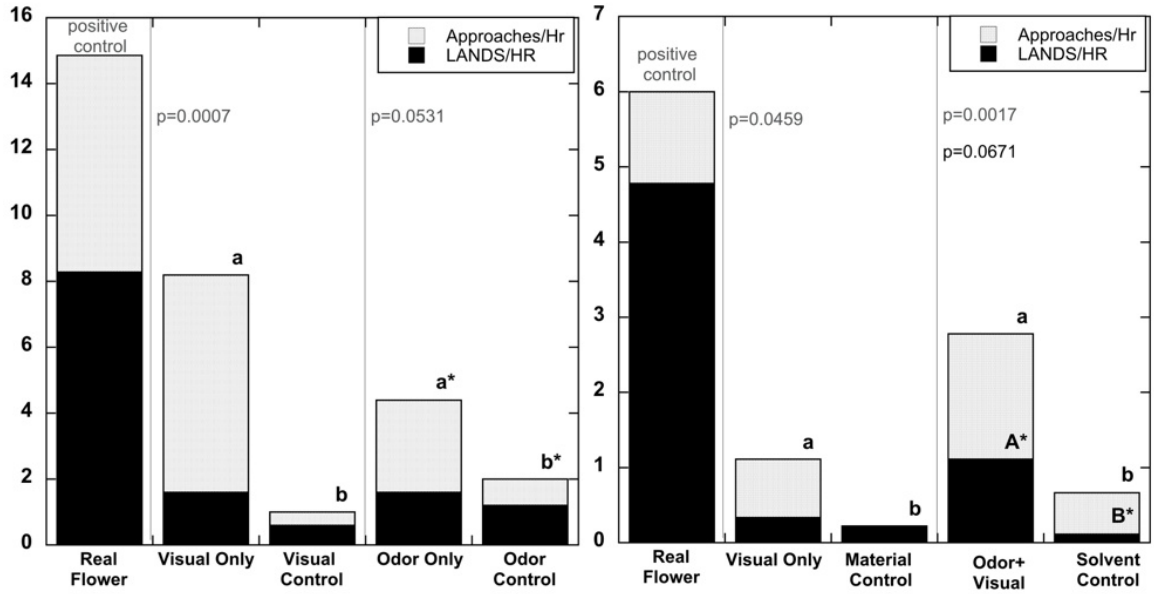

Figure 3.3. a) Floral Deconstruction: ***Both odor and visual cues attract floral visitors.*** Approaches (full bars) to the array depended on treatment. Individual contrasts show differences between the 'masked' treatments and their negative controls; 'Visual Only' vs. 'Visual Control' was significant $F_{1,33.7}$=14.05; p=0.0007; 'Odor Only' vs. 'Odor Control' was marginally so $F_{1,33.7}$ =4.02; p=0.0531. Neither actual landing rates (black portion of the bars) nor visit duration (data not shown) showed significant differences between the 'masked' treatments and their controls. N=ten 30 min. time blocks (5 hours total); N=420 flies. **b). Floral Reconstruction*: Odor extracts added to a visual model stimulate landings.*** Approaches (full bars) depended on treatment. Individual contrasts show that the artificial flower treatments were more attractive than their negative controls; 'Visual Only' vs. 'Material Control' $F_{1,68}$=4.13; p=0.0459; 'Odor+Visual' vs. 'Solvent Control' $F_{1,68}$=10.62; p=0.0017. Landing rates (black portion of the bars) depended on treatment as well (whole model $F_{4,68}$=5.41; p=0.0008); the 'Odor+Visual' treatment evoked more 'marginally' more landings than its negative control ($F_{1,68}$=3.46; p=0.0671). Visit duration (data not shown) showed no significant differences between the treatments and their controls. N=eighteen 30 min. time blocks (9 hours total). N=97 visitors.

Bars (within a panel) that share a letter are not significantly different from each other (direct contrasts; '*'indicates marginal significance). No treatment displayed the attractiveness of the 'Real Flower' and the 'Real flower' was included as a positive control and not included in the *a priori* hypothesis testing. Error bars were omitted due to the stacked columns. Statistical tests were performed on log-transformed data, but raw data is presented for clarity of interpretation.

Chimeras: both calyx and labellum play role in attraction

The Real Flower was visited at significantly higher rates and visitors stayed longer than at the Artificial Flower (Figure 3.4). Both chimeric treatments were intermediate in terms of landing rate (whole model $F_{3,44}$ =4.55; p=0.0073) (Figure 3.4a) . While visit duration to the Real Labellum Chimera was intermediate, the Real Calyx Chimera actually retained visitors longer than the Artificial Flower (whole model $F_{3,32}$ =4.23; p=0.0126) (Figure 3.4b). The landing rate data are based on N=thirteen 30 min. time blocks (6.5 hours total) and 359 visitors. The visit duration data are based N=nine 30 min time

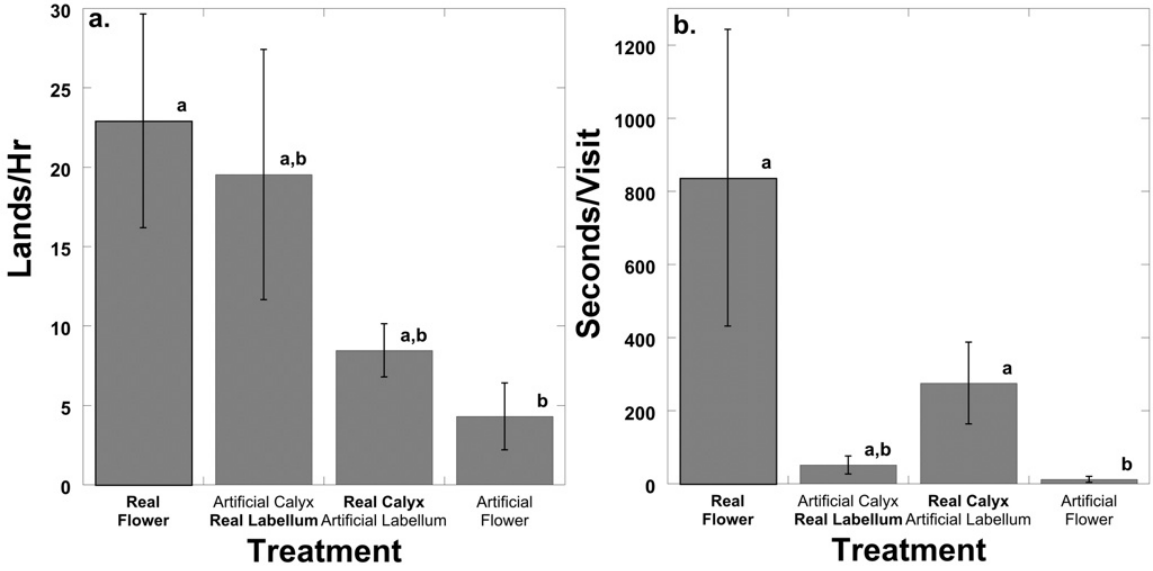

Figure 3.4. Chimeras*: Both calyx and labellum play a role in attraction and retention.* a) Landings depended on treatment ($F_{3,44}$ =4.55; p=0.0073). Visitation to both 'chimera' treatments (Artificial Calyx/ Real Labellum & Real Calyx/Artificial Labellum) was intermediate to both the Real Flower and the Artificial Flower (Tukey's HSD). N=thirteen 30 min. time blocks (6.5 hours total). N=359 visitors. **b) Visit duration** also depended on treatment ($F_{3,32}$ =4.23; p=0.0126). Both 'chimera' treatments were intermediate to both the Real Flower and the Artificial Flower. N=nine 30 min time blocks (4.5 hours total). N=77 visitors. Bars that share a letter (within each graph) are not significantly different from each other (Tukey's HSD). All error bars represent one standard error. Statistical tests were performed on log-transformed data, but raw data is presented for clarity of interpretation.

blocks (4.5 hours total) and 77 visitors – four replicates were omitted from this analysis. Due to the overwhelming number of visitors to the array, it was impossible to accurately keep track of visit duration for each individual fly.

The transitions diagram (Figure 3.5) supports the visit duration data (Figure 3.4b). Visits to the Real Flower are more complex, with more intra-floral movements than the

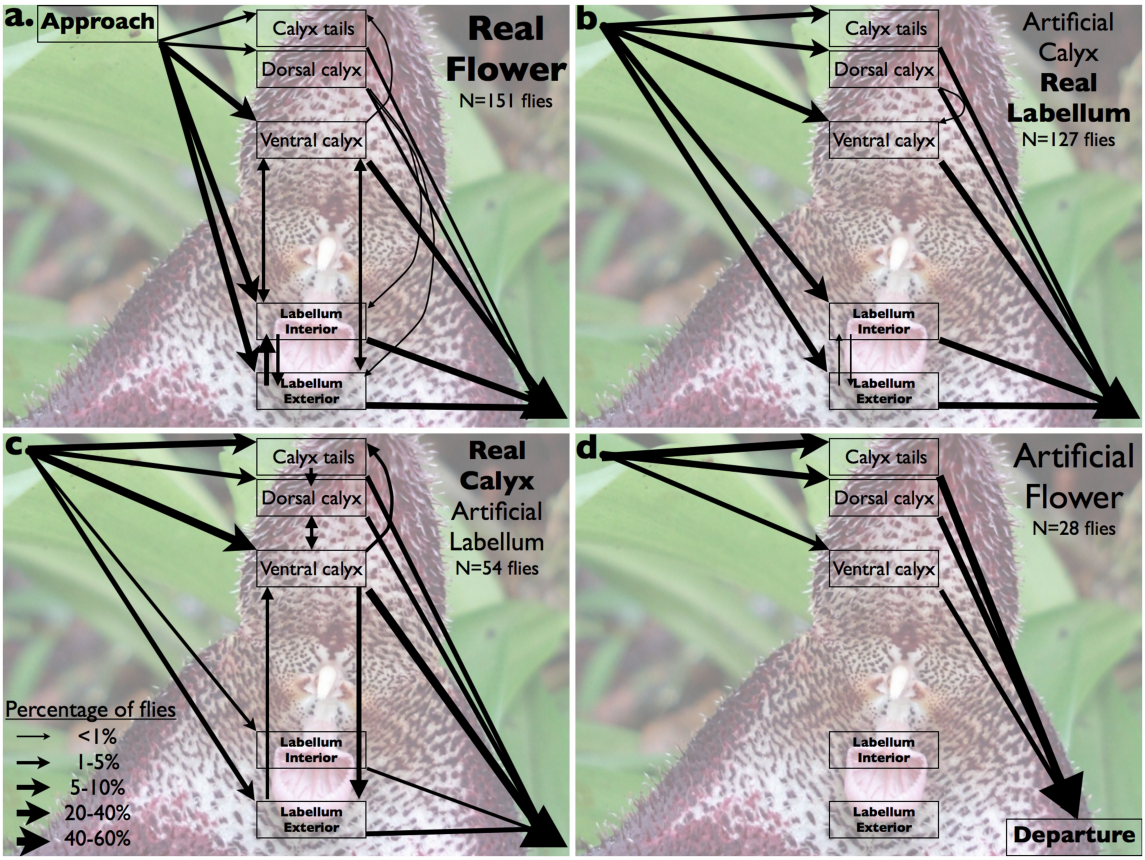

Figure 3.5. Chimeras: ***Treatment affects both attractions and intra-treatment transitions.*** The percentage of flies to make each transition within each treatment is illustrated. **a) Real Flower:** Most approaches directed toward the labellum (69.5%) or ventral calyx (25.8%). Intrafloral transitions were made by 13.3% of visitors. **b) Real Labellum Chimera**: Most approaches directed toward the labellum (62.2%) or ventral calyx (23.6%). Intrafloral transitions were made by 1.6% of visitors. **c) Real Calyx Chimera:** Most approaches directed toward the ventral calyx (48.1%) or the dorsal calyx (14.8%) and the calyx tails (27.8%). Intrafloral transitions were made by 16.7% of visitors. **d) Artificial Flower:** Approaches directed wholly toward the calyx tails (53.6%), the dorsal calyx (35.7%) and the ventral calyx (10.7%). Intrafloral transitions made by 0% of visitors. Photo © T. Policha.

other treatments (in particular to and from the labellum), with a substantial proportion of flies making these intra-floral transitions (13.3% of visitors). The unscented Artificial Flower shows the least amount of intra-floral movement (0% of visitors). Also no visitor to the Artificial Flower ever moved to the labellum. The Real Labellum chimera had more visitors than the Real Calyx Chimera, and more visitors to the labellum, but few intra-floral transitions (1.6% of visitors), and no transitions between the calyx and the labellum. A higher proportion of visitors to the Real Calyx Chimera transitioned between floral organs (14.8% of visitors), which is consistent with the visit duration data, visiting flies spent more time making intra-floral movements on this treatment.

In terms of preference for first-landing location across treatments we found significant differences (Figure 3.5 & Figure 3.6, and Table 3.1). The treatments with real labella (the Real Flower and the Real Labellum Chimera) evoked more landings to the labellum (Real Flower: $G=106.05$, $df=4$, $p<0.0001$; Real Labellum Chimera: $G=45.49$, $df=4$, $p<0.0001$). The treatments without real labella (the Artificial Flower and the Real Calyx Chimera) evoked more landings to the calyx (Artificial Flower $G=37.41$, $df=4$. $P<0.0001$; Real Calyx Chimera $G=38.03$, $df=4$, $p<0.0001$). In the Real Calyx Chimera

Table 3.1. First-Landing Locations Across Real, Artificial and Chimeric Flower Treatments

Treatment	Calyx Tails	Dorsal calyx	Ventral calyx	Exterior labellum	Interior labellum	Total Lands /Treatment
Real flower	1.99%	2.65%	25.83%	34.44%	35.10%	**151**
Artificial calyx **Real labellum**	6.30%	7.87%	23.62%	37.01%	25.20%	**127**
Real calyx Artificial labellum	27.78%	14.81%	48.15%	7.41%	1.85%	**54**
Artificial Flower	53.57%	35.71%	10.71%	0%	0%	**28**

First landing location, and indeed landings overall depended on the presence of a real labellum. The whole model test from the combined G-test was significant ($G=160.31$, $df=12$, $p<0.0001$), with the notable non-significant subsets being: the 'Real Flower' and the 'Real Labellum Chimera' (ACRL) across all floral parts ($G=9.61$), and the 'Artificial Flower' and the 'Real Calyx Chimera' (RCAL) also across all floral parts ($G=19.68$).

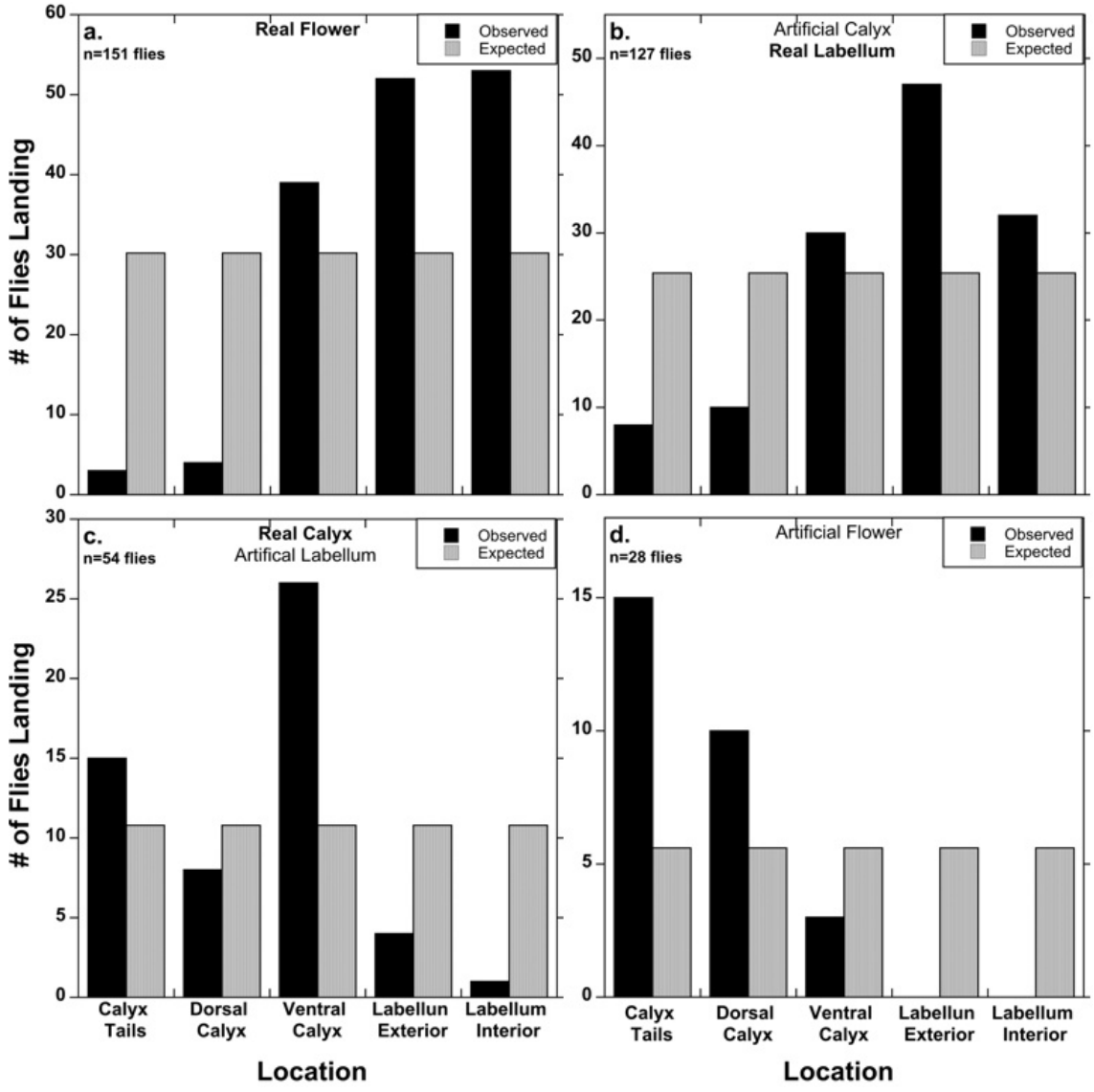

Figure 3.6. Chimeras: ***Landing preference depends on treatment.*** Expected frequencies are derived from the null hypothesis of random landings. Goodness-of-fit G-tests show deviation from the expected distribution in all cases, but the preferred region depends on the treatment. **a) Real Flower:** Regions closest to the column [(ventral calyx, and labellum (exterior and interior)] were preferentially approached G=106.05 df 4, p<0.0001. **b) Artificial Calyx Real Labellum:** a similar pattern was seen; points closest to the column were preferentially approached. G=45.49 df 4, p<0.0001 **c) Real Calyx Artificial Labellum:** showed strong preference for the various parts of the calyx, with the labellum being largely avoided. G=38.03 df 4, p<0.0001 **d) Artificial Flower:** landings to this treatment were exclusively to eht calyx, and particulary to regions most distal to the column, the dorsal side and the tails. G=37.41 df 4, p<0.0001.

the bulk of visits were to the ventral side of the calyx (closer to the labellum and the column). In the Artificial Flower most of the visits were to parts of the flower (calyx

tails) most distal to the reproductive structures and none were to the labellum (Figure 3.6, Table 3.1).

Not only did landings to the labellum drop off precipitously without a real labellum, but landings overall depended on its presence. The whole model test from the combined G-test was significant ($G=160.31$, $df=12$, $p<0.0001$), with the notable non-significant subsets being: the Real Flower and Real Labellum Chimera across all floral parts ($G=9.61$), and the Artificial Flower and Real Calyx Chimera also across all floral parts ($G=19.68$) (Table 3.1).

Visual cues: contrast & pattern more important than color

A total of 44 visitors were observed in this experiment. The contrasting treatments ('spotted' and 'striped') were more attractive than the solid colored treatments (Figure 3.7) (whole model (not including the real flower) $F_{3,15}=4.59$; $p=0.0179$). Visitation to the 'striped' treatment was significantly higher than to the solid treatments, while the 'spotted' treatment was intermediate between the 'striped' and the solid treatments. There were no significant differences between treatments in terms of visit duration.

Volatile chemistry: mushroom volatiles are novel to* Dracula *labella

Volatile fragrance bouquets differered significantly among the three groups: pleurothallid orchids, *D. lafleurii,* and mushrooms (Figure 3.4a, Appendix B). The *Dracula* orchids have an odor profile that is intermediate, sharing floral scents (2-phenylethanol, benzyl alcohol, and methyl salicylate) with other pleurothallid orchids and the fungal 1-octen-3-ol with the mushrooms (MRPP $A=0.33$; $p<0.0001$, see Figure 3.4a for axis loading). This pooled result conceals a more important pattern, when we dissected the flowers we found discrete volatile emission patterns (MRPP $A=0.46$;

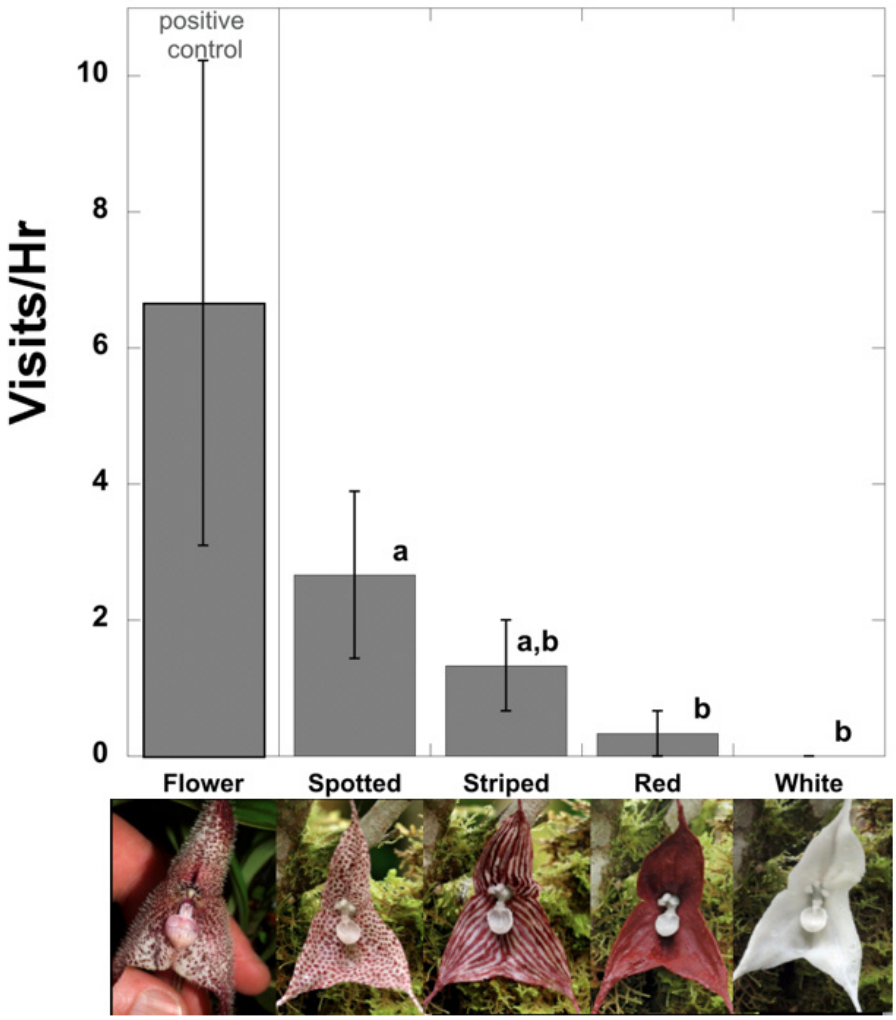

Figure 3.7. Visual Cues*: Contrast and pattern are more important than color.* Landing rates depended on treatment (whole model (not including the real flower) $F_{3,15}=4.59$; p=0.0179); visitation to the treatments with contrasting patterns was higher than to the solid colors. Visit duration data (not shown) shows the exact same pattern ($F_{3,15}=3.94$; p=0.0296). N=six 30 min. time blocks (3 hours total). N=44 visitors. Bars that share a letter are not significantly different from each other (Tukey's HSD-positive control excluded). All error bars represent one standard error. Statistical tests were performed on log-transformed data, but raw data is presented for clarity of interpretation.

p<0.0001, see Figure 3.4b for axis loading). The labellum of *D. lafleuri* produces the fungal 8-carbon alcohol, whereas the aromatic compounds common to other orchids are localized to the column, suggesting an olfactory division of labor (Figure 3.4b, Appendix B). The calyx did not have much odor at all (small peak areas – data not shown) despite being the largest and brightest colored organ. When volatiles were detected, they tended

to be either wound volatiles such as E-2-hexanol and Z-3 hexanol or they shared the methyl salicylate note found in the flower's column – to which they are in close proximity. The lateral petals did not contribute much to the overall volatile profile (only three out of 22 samples registered any volatiles), but when they did, they contained the floral compounds 2-phenyl ethanol and benzyl alcohol, while the column typically showed a strong methyl salicylate peak and some benzyl alcohol. The mushroom-scented compound 1-octen-3-ol was found exclusively in extracts of the labellum.

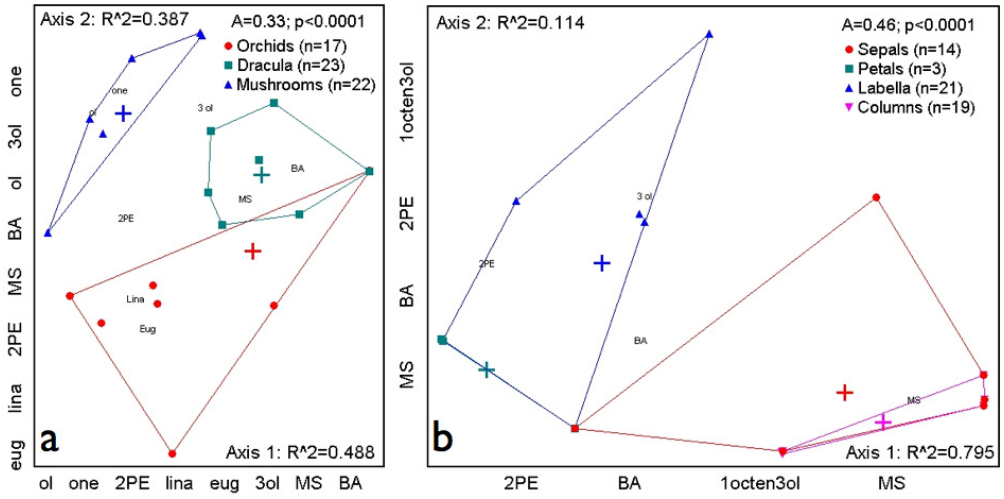

Figure 3.8. a) ***Mushroom volatiles are novel to*** **Dracula**. Nonmetric Multidimensional Scaling ordination results for the major volatile components of *Dracula lafleurii*, related, but non-mushroom-mimicking pleurothallid orchids and co-occurring mushrooms. 2PE, BA, Eugenol, linalool and MS are typical floral volatiles and were only found in the pleurothallids and the *Draculas*, while 1-octen-3-ol, 3-octanol and 3-octanone are typical mushroom odors and were only found in the mushrooms, with 1-octen-3-ol also appearing in *Dracula lafleurii*. Pair-wise comparisons indicate that all groups are significantly different. **b) Fungal volatiles are produced by the labellum**. Nonmetric Multidimensional Scaling ordination results for the major volatiles produced from different parts of the *Dracula* flowers. The sepals did not have much odor, the petals contained the floral compounds 2-phenyl ethanol and benzyl alcohol, the column typically showed a strong methyl salicylate peak and the mushroomy 1-octen-3-ol was found almost exclusively in the labella. Pair-wise comparisons indicate that all groups are significantly different except sepals and columns (p=0.457, not corrected for multiple comparisons). The '+' sign indicates the centroid for each group, text within figure indicates the x,y mean position of each chemical species. ('BA'=benzyl alcohol, 'MS'=methyl salicylate, and '2PE'=2-phenylethanol, 'ol'=3-octanol, 'one'=3-octanone, '3ol'=1-octen-3-ol).

DISCUSSION

Our manipulative experiments clearly demonstrate that both visual and olfactory aspects of the *Dracula lefleuri* floral phenotype are important in the attraction of pollinating flies (Figure 3.3, Figure 3.4 & Figure 3.7). In the deconstruction experiment (Figure 3a), both visual and olfactory cues were sufficient to elicit approaches by flies, but neither was adequate to elicit a landing. In the reconstruction experiment we saw a similar result in terms of approaches (Figure 3.3b), with both the 'Visual Only' and the 'Odor+Visual' treatments being approached more than their controls. However, it was only the 'Odor+Visual' treatment that actually induced more landings to the treatment than to its negative control. These data suggest a synergistic effect in the signaling phenotype.

The combination of traits may be important in terms of acting over different spatial scales or by affecting visitor retention in addition to just attraction. Alternatively, we know that *D. lafleurii* is visited by at least 17 different species of small drosophilid fly. These diverse visitors may have distinct life-history strategies and distinct perceptual biases and be responding to different aspects to the phenotype (Leonard et al. 2011). The ability to attract a diverse assemblage of mushroom-associated flies may be beneficial given the ephemeral nature of fungal fruiting bodies. Any degree of specialization in mushroom-visiting flies may cause significant temporal turnover in the community that is available to serve as pollinators, promoting generalism in a mushroom-mimic.

Whatever the case, once at the flower, the visitor needs to stay there long enough to move pollen in order for the plant to experience a benefit. It has been shown, at least in species without aggregated pollen, that longer visits result in both greater pollen removal

(Harder and Thomson 1989), and greater pollen deposition (Thomson and Plowright 1980). In this system, where pollen removal and pollen deposition are exceedingly rare (pers. obs.), the more time spent by a visitor may lead to a higher probability of pollen removal/deposition. In none of our experiments did we see significant differences for visit duration, with the exception of the chimera study. In this experiment the Real Calyx Chimera treatment had similar visit duration to the Real Flower and significantly longer visits than the Artificial Flower. One explanation for the longer visit duration in the absence of olfactory cues, or yeast covered labella is visitor confusion. There was a relatively high proportion of visitors that made intra-floral transitions in the Real Calyx Chimera treatment (Figure 3.5), visitors traveled between all points on the calyx, and from the calyx to the labellum and back again. They were more active, but less directed than on the treatments that included a real labellum.

Our volatile results (Figure 3.8b) show that the majority of the fragrance in these flowers is concentrated either in the labellum (mushroom volatiles) or in the directly adjacent column (floral aromatics). All of these volatiles were included with the 'labella' in the construction of the chimeras, as the entire corolla as well as the column the ovary and the peduncle were necessary to support the calyx (Figure 3.2c). This result suggests that odor is an important attractant over a longer distance, but that something else about the calyx may be important for visitor retention. Since our fabricated flowers did include the color/visual cues, this may suggest a tactile or gustatory/contact chemoreceptive component of visitor retention that we were unable to address in the current study. The calyces are also variously pubescent, possibly focusing visitors toward the column or otherwise promoting longer visit durations. Alternatively, we know that the calyces are

often damaged by herbivory at our field sites (pers. obs.), so herbivore defense may be another explanation for the hairs, as non-pollinating agents can also function as selective forces in flower evolution (Strauss and Whittall 2006).

The color/contrast experiment also indicates that visual cues play an important role in eliciting visitation from the known pollinators. These data further suggest that the attractiveness of contrasting patterns seems to act at different scales. The 'white' artificial flower treatment, which provided the highest level of contrast against the background vegetation, received the least number of visits, yet the two treatments that displayed finer scale contrast (50% red and white 'spotted' or 'striped') had visitation rates well above the solid treatments (Figure 3.7). It may be that smaller scale spots or stripes provide the contrast at a scale that the flies can perceive and they simply don't see the white or red flowers. The pattern of the spots and or stripes may be important too. There is a literature that supports linear corolla or calyx markings as guiding fly behavior toward the center (reproductive parts) of flowers (Johnson and Dafni 1998, for example).

Another plausible role for the speckled [Figure 3.1, Supplemental Figure S3.1 (Appendix A)] calyces is to exploit the lekking behavior ((Grimaldi 1986, Burla 1990) of these flies by presenting floral decoys. We know that both intra- and inter specific aggregation are common in these dipteran taxa (Jaenike and James 1991, Jaenike et al. 1992), and the small dark spots on a light background may look like other flies serving to attract additional individuals, functioning as floral decoys (Johnson and Midgley 1997, pers. obs., and pers. com. D. Grimaldi). We occasionally observe "swarming" (>180 visits/hr) in which large groups of flies aggregate, mate, and engage in territorial combat at the flowers. A phenomenon also seen by *Zygothrica* spp. on mushrooms (Grimaldi

pers. comm.) The sepaline spots may be important for the initial attraction of these large groups. At least some of the species of flies visiting *Dracula* flowers are also found on mushrooms, where they are known to court, mate, and (in some species) breed (Grimaldi pers. com. and unpub. data.)

Interestingly, the calyx of *Dracula lafleurii* displays the splotchy maroon coloration typical of sapromyophilous or 'carrion' flowers (Jürgens et al. 2013), but without the attendant foul odor profile. The carrion pollination syndrome is often associated with brood-site mimicry (Urru et al. 2011). One way that mimicry could have evolved is that the perceptual bias towards visual cues of the calyx may have preceded the fungal mimicry. Shuttleworth and Johnson (2010) argue that scent is a sufficient cue to cause pollinator shifts from wasp to carrion-seeking flies in South African *Eucomis* lilies. One possibility may be that the fungal scent profile (which is not unknown, but is exceedingly rare within *Masdevallia* (Kaiser 1993b)), was sufficient to cause shifts into a fungal-associated pollinator space, perhaps leading to the radiation of the genus *Dracula*. There are *Masdevallia* species (the former generic home of *Dracula* (Luer 1978)) that have taken the carrion-motif all the way to producing carrion fragrances (ie. *M. caesia* & *M. elephanticeps* with butryic and isovaleric acids as the dominant fragrance (Kaiser 1993b)) although their pollination ecology has not been studied.

Dracula flowers have long been suspected of being Batesian mimics (Vogel 1978) in part due to the lack of a visible nectar reward, which was confirmed in *D. lafleurii* with Combur® strips by Endara et al. (2010). Floral variability is predicted in Batesian or deceptive mimicry systems one as a way to slow learning in visitors (Moya and Ackerman 1993, Roy and Widmer 1999, Salzmann et al. 2007). *Dracula lafleurii* fits

this pattern, showing considerable variation in both size (calyx area = 411-1623mm^2; labella area 16-97mm^2) and the degree of contrast in coloration [percent pigmentation in the calyx = 38-93%; n=22 flowers [Supplemental Figures S3.1 and S3.2 (Appendix A)]. This is also predicted due to the generalized nature of the mimicry, as without a specific model there is a range of phenotypes that may be considered attractive. Our experiments show that the pollinators respond to variation in color and pattern (Figure 3.7).

Two main features of the volatile components of the phenotype stand out. Firstly, that the volatile compound responsible for the 'mushroomy' odor in *Dracula lafleurii* is chemically identical to that in the mushrooms themselves. This has been shown for other *Dracula* species as well, both by the current authors (Figure 3.8b and Appendix B), as well as by Roman Kaiser (1993a, 1993b, 2006). The 8-carbon volatile 1-octen-3-ol is the character-bearing olfactory note associated with cultivated *Agaricus* mushrooms (Buchbauer et al. 1993, Combet et al. 2006). Secondly, the emission of this fungal volatile is restricted to the only visibly mushroom-like part of the *Dracula* flower, the labellum (Figure 3.8b). Additionally, by sampling other co-occurring and closely related (sister group) orchids (*Masdevallia* spp.etc.) we were able to demonstrate the novelty of this mushroom odor within the *Dracula* at our field site. This is suggestive that these volatiles are selected for rather than simply a result of phylogenetic constraint.

1-octen-3-ol and other 8-carbon compounds are known to act as insect attractants when produced by fungi, attracting both fungivorous and fungivore-predator species (Pierce et al. 1991, Faldt et al. 1999, Combet et al. 2006), although both concentration and enantiomeric configuration seem to influence the effects on behavior (Cammaerts and Mori 1987). 1-octen-3-ol has also been implicated in herbivore defense and may play

a similar role *D. lafleurii*, either secondarily to attraction or vice versa (Wood et al. 2001). The other volatiles produced by *Dracula* orchids are typical floral compounds (Knudsen et al. 2006, El-Sayed 2012) found in many orchid lineages worldwide (Kaiser 1993a), thus their detection in the other sampled orchids was not surprising.

The volatile bouquet of *Dracula lafleurii*, while possessing components of both orchid and mushroom profiles, did not completely overlap with either group (Figure 3.3a). Unique to the other orchids were the common floral volatiles eugenol and linalool and unique to the mushroom profile were the fungal volatiles 3-octanol and 3-octanone. While these two 8-carbon compounds were absent from the *D. lafleurii* bouquet they were both present in other *Dracula* species assayed during the course of this study (Appendix B). Both 3-octanol and 3-octanone were found in *D. morleyi* and 3-octanol was present in samples of *D.* cf. *pubescens*. Both of these other species also contained the unsaturated 1-octen-3-ol (all in the labellum). In mushrooms, 1-octen-3-ol is the most abundant 8-carbon fungal aroma (Combet et al. 2006), and may function in part as a signaling molecule, correlated with events such as sporulation (Faldt et al. 1999), and spore germination (Chitarra et al. 2004). In terms of prevalence and abundance 1-octen-3-ol is a key ingredient of the fungal volatile motif (Tressl et al. 1982, Combet et al. 2006), and is therefore predicted to be a common aspect in signal convergence and the evolution of fungal mimicry.

It is clear that the integration of both visual and olfactory cues is critical to the success of *Dracula lafleurii* in attracting visitors. The same phenomenon has been found in other pollination systems, with each component on its own eliciting a subset of necessary behaviors, but both being required for complete pollinator attraction and

visitation (Raguso and Willis 2002), so it is the cross-kingdom mimicry in both visual and chemical signals that makes this system remarkable. We also suspect that there may be multiple layers to the mimicry. The fact that the part of the flower that looks like a mushroom is where the fungal volatiles are concentrated is intuitively satisfying, yet the large showy calyx also clearly plays a role in visitor attraction. Are they simply serving as umbrellas in an extremely rainy habitat? Are they acting as decoys, giving the visual appearance of aggregating flies displaying at a lek, or are they playing on some other bias in the fly's visual, gustatory, or tactile senses? Further study on fly behavior at these and mushroom hosts may further elucidate the role that the calyx plays in this system.

BRIDGE TO CHAPTER IV

In chapter III we demonstrated discreet contributions of the visual and olfactory cues in visitor attraction. Both aspects of the signaling phenotype are important in visitor attraction, however we found a synergistic effect, whereby both elements together evoke more landings than either cue on its own. By using chimeras composed of real and artificial flower parts in experiments we were able to suggest relative importance to different floral organs in both visitor attraction and visitor retention. Our chemical analyses demonstrated that the fungal volatile, 1-octen-3-ol, is produced exclusively by the labellum of these *Dracula lafleurii* flowers. The labellum is also the part of the flower that is mushroom-like in appearance. The large and showy calyx, while playing a demonstrated role in stimulating visitation, does not obviously contribute to the overall 'mushroom' signal. Its existence may indicate an additional component to the sensory display. It may function to attract a larger community of flies, or simply be a relic of

evolutionary constraints. In chapter IV we undertake a detailed identification of these visitors. We compare species found on *Dracula* flowers with species found on mushrooms, and analyze the overlap in the communities visiting each substrate. We present evidence for individual flies moving between the two substrates, as well as measure the display of certain behaviors on both the *Dracula* flowers and mushrooms. To understand resource use on the different substrates by these flies and to address the hypothesis of brood-site mimicry, we also implemented a rearing program. The results from this chapter show significant convergence in the communities visiting the two substrates, but also reveal some novelty. These data suggest that these diverse visitors may have distinct life-history strategies and perceptual biases, and may be responding to different aspects of the phenotype. The multimodality of the signals demonstrated in chapter III may function to broaden the community of visitors to *Dracula* flowers.

CHAPTER IV

DOES *DRACULA* ALSO APPEAR AS A MUSHROOM? SUBSTRATE UTILIZATION BY CLOUD FOREST DROSOPHILID FLIES

Rocío Manobanda, Adrian Troya, Bryn Dentinger, Bitty Roy and myself coordinated the field collection of all insect specimens. Bryn Dentinger, Rocío Manobanda and I designed the rearing program. Bryn Dentinger and I designed and optimized the protocol for the DNA barcoding, though much of the lab work was performed by Ashley Ludden. Bitty Roy, Ashley Ludden and I edited all of the sequence data. Field observations and insect videos were captured by Adrian Troya and myself. David Grimaldi conducted all of the morphological insect identifications below the level of family, and provided insightful text for the Discussion section. Ashley Ludden and Bryn Dentinger contributed text for the Methods section. I performed all of the statistical analysis and wrote the chapter with editorial input from these co-authors.

INTRODUCTION

Dracula orchids have long been thought to be mushroom mimics, attracting small, mushroom-visiting flies to pollinate them. Early speculation suggested fungus gnats (i.e. Mycetophiloidea) may be the pollinators (Vogel 1978). However, observation of the flowers has revealed that the main floral visitors are mushroom-associated flies in the family Drosophilidae (Endara et al. 2010). Both the hypothetical fungus gnats and the actual *Dracula*-visiting drosophilids [mostly in the genera *Zygothrica* and *Hirtodrosophila* (Endara et al. 2010)] are either known to use fungi as breeding sites or as

congregation-rendezvous-mating sites. This leads to the hypothesis that *Dracula* flowers act as brood-site mimics, which effectively imitate a substrate that visitors would normally utilize for oviposition and larval development (Urru et al. 2011). This deceptive pollination strategy is known from a diverse range of plant families, including the Araceae, Aristolochiaceae, Asclepiadaceae, and Orchidaceae (Atwood 1985, Miyake and Yafuso 2003, Jersáková et al. 2006, and references therein, Trujillo and Sersic 2006, Ollerton et al. 2009).

To test the brood-site hypothesis it is necessary to determine what the visitors are to both the mushrooms and the orchids, and determine where they breed. To date the dipteran community that visits *Dracula* flowers has not been well characterized despite recent contributions by Endara et al. (2010). Not only is the visitor community relatively unknown, but it is completely unknown whether these species concomitantly visit mushroom fruiting bodies, and indeed if that is where they breed. Most brood site records for *Zygothrica* are actually from flowers (Grimaldi 1987, dos Santos and Vilela 2005).

This sets up an interesting framework in the context of mimicry theory, with its dichotomy between Batesian and Müllerian mimicry. Batesian mimicry, which is based on deception and a subsequent loss of fitness in the dupe is the manner in which most brood-site mimicries are achieved (Roy and Widmer 1999, and references above). However, if the main visitors truly are members of the speciose genus *Zygothrica,* which are known to breed in flowers, then this could be suggestive either of a more Müllerian system with its convergent phenotypes and rewards (Roy and Widmer 1999), or perhaps show that this long-held anomaly of natural history (Vogel 1978, Jersáková et al. 2006) is

not in fact a mimetic system and that the floral visitors are actually breeding in the flowers themselves.

An added complexity is that these orchids grow in regions that are widely recognized to be some of the most biodiverse on the planet (Myers et al. 2000). This means that not only do we find several species of *Dracula* growing in sympatry, but that they may be visited by a diverse community of flies. Endara et al. collected over 15 species of flies from just two species of *Dracula* in 2010. If *Dracula* is in fact a mushroom-mimic, then the range of potential mushroom models may also be hyper-diverse (pers. obs. and Dentinger et. al. in prep).

Beginning to make sense of this ecological network requires a multi-disciplinary approach. Using a combination of morphological, molecular, and multi-variate statistical techniques, in addition to field observations and collections, we specifically address the following questions: 1. Do *Dracula* spp. attract the same fly species as co-occurring mushrooms? 2. Are the fly communities found on *Dracula* similar to those found on co-occurring mushrooms? 3. Do the same individual flies move between *Dracula* spp. and co-occurring mushrooms? 4. Do drosophilid visitors show non-random host use amongst substrates? 5. Do drosophilid visitors to mushrooms, as well as *D. felix* and *D. lafleurii*, display similar patterns of behavior across the three substrates? and 6. Are drosophilids breeding in mushrooms and *Dracula* orchids?

Answers to these questions will not only inform our perspective on *Dracula* pollination, but also expand our understanding of mimetic relationships and pollination networks in communities of exceptionally high biodiversity.

METHODS

Study site

The majority of fieldwork was performed at Bosque Protector Reserva Los Cedros, which is located between 1,250 and 2,200 m elevation on the western slope of the Andes in northwestern Ecuador (00°18'31.0"N, 78°46'44.6"W). This private reserve protects 6,900 hectares of montane cloud forest, 5,800 hectares of which is primary forest. The reserve is a buffer zone for the 300,000 hectare Reserva Ecológica Cotacachi-Cayapas, and is part of the Chocó phytogeographical zone, recognized as one of the most biologically diverse habitats on earth (Myers et al. 2000). The reserve experiences an average annual rainfall of 2,903 mm (SE=186.41) (José DeCoux pers. com.). Our studies were conducted in 2008, and 2010-12, during the local rainy season (January-March) when the *Dracula* orchids were in peak bloom. Additional insect collections were made at nearby locales (two collections at Reserva Orquideológica El Pahuma (near Quito) resulting in 8 individuals, and a single collection of 17 individuals at Cabañas Armonia y Jardin de Orquídeas in Mindo).

Dracula *Luer*

The genus *Dracula* is part of the diverse Neotropical subtribe Pleurothallidinae (Epidendroideae) which accounts for 15-20% of the species diversity in the Orchidaceae (Pridgeon et al. 2001). *Dracula* orchids (~125-150 spp.) are epiphytes in mature forests ranging from southern Mexico to Peru, reaching their peak diversity in the wet forests that cover the slopes of the western Andes in Colombia (>60 spp.) and Ecuador (>45 spp.) (Luer 1993, Jorgensen and León-Yanez 1999).

Dracula species are generally thought to be fly-pollinated, despite a paucity of empirical documentation (Van der Pijl and Dodson 1966, Pridgeon et al. 2001, Van der Cingel 2001). Herein we have collected insects and made observations at six different species of *Dracula* (Appendix C) including *D. chiroptera* Luer & Malo (n=1), *D. felix* (Luer) Luer (n=41), *D. lafleurii* Luer & Dalström (n=19), *D. morleyi* Luer & Dalström (n=6), *D. cf. pubescens* (most likely an undescribed species, G. Meyer pers. comm.) (n=6), and *D. sodiroi* (Schltr.) Luer (n=1). We focus primarily on two species, *D. felix* and *D. lafleurii* (Figure 4.1), which represent a range of floral morphology within the genus, although collections from the other species are included in some analyses. The small (8-9 mm sepals), cup-shaped flowers of *D. felix* (Figure 2.1) with a shallowly concave labellum (4.5x2 mm) and tiny petals (3x1.25 mm) are presented singly on individually peduncles (Luer 1993). Numerous flowers (up to 50) are produced simultaneously on each plant, with flowering concentrated in the wet season (Dec-Mar). *Dracula felix* tend to congregate on the ridge tops (~1,650 m) where they can be locally abundant (pers. obs.). In contrast, *D. lafleurii* produces pendant, umbrella-like flowers (Figure 4.1) with subglobose labella on descending, few-flowered racemes (~1-5 racemes at a time). *Dracula lafleurii* sepals are much larger (25-30 mm), as are the petals (3x2 mm) and labellum (11.5x9 mm) (Luer 1993). Plants of *D. lafleurii* continue to flower throughout the year. Our work with *D. lafleurii* was done at the only known site of these plants on the banks of the Los Cedros river at ~1,300m elevation (Luer 1993). Both *D. felix* and *D. lafleurii* were assayed by Endara et al. (2010) for nectar production, with no detectable levels observed.

Notably, the flowers of *Dracula sodiroi* are so distinctly different from the other species in our study, and indeed within the genus, that it occupies a subgenus that bears its name, *Sodiroa*, which it shares with only one other species, *D. erythrocodon*. The flowers of *D. sodiroi* are not obvious mushroom-mimics, displaying a narrow, ligulate labellum (Luer 1978) and a tubular, bright-orange calyx [Supplemental Figure 4.1 (Appendix A)] that is reminiscent of a hummingbird pollination syndrome.

The four most common *Dracula* spp. in this study have been vouchered with specimens deposited at the Herbario Nacional del Ecuador (QCNE) in Quito (*D. felix*, R. Manobanda #332,334; *D. lafluerii*, R. Manobanda #331; *D. morleyi*, R. Manobanda#333; *D.* cf. *pubescens*, R. Manobanda #330).

Figure 4.1. Umbrella-like calyx of *Dracula lafluerii*, a pollinium-carrying *Zygothrica* sp. on a mushroom, and a *Zygothrica* sp. extending its proboscis on the labellum of *D. lafleurii*. a) a mixed flock of drosophilid visitors sheltered within a *D. lafleurii* flower. b) A *Zygothrica* fly carrying an orchid pollinium while semaphoring on the top of a mushroom pileus (*Polyporus craterellus*, RLC 717). c) *Zygothrica* sp. extending its proboscis on a *D. lafleurii* labellum, we have shown that these labella support a yeast community that may provide nutrition to these flies (McAlpine 2012). a & c ©B. Roy, b ©T. Policha.

Mushrooms

We collected adult insects visiting ~90 different fleshy, ephemeral mushrooms representing a phylogenetically diverse assemblage. Most of these (61) belong to families of Agaricales: Agaricaceae (7), Cortinariaceae (2), Entolomataceae (3), Hygrophoraceae

(3), Inocybaceae (3), Lycoperdaceae (1), Marasmiaceae (12), Mycenaceae (14), Physalacriaceae (3), Pleurotaceae (3), Pluteaceae (4), Psathyrellaceae (1), Strophariaceae (1), Tricholomataceae (4). In addition, insects were also caught from mushrooms in the Boletinellaceae (1) (Boletales), Polyporaceae (8) (Polyporales), and undetermined families (18).

Insects were also reared from 45 mushrooms belonging to the Agaricales [Agaricaceae (2), Cortinariaceae (1), Marasmiaceae (13), Mycenaceae (8), Physalacriaceae (1), Pluteaceae (2), Polyporaceae (8), Pterulaceae (1), Tricholomataceae (2), Auriculariales [Auriculariaceae (1)], Boletales [Boletinellaceae (1)], and undetermined families (4). One Ascomycota [Xylariaceae (1)] was also represented.

Specimen vouchers are deposited at the Herbario Nacional del Ecuador (QCNE) and Royal Botanic Gardens, Kew (K). See Supplemental Figure S4.2 (Appendix A) for photos of some representative specimens.

Insect collections

To identify the floral visitors and determine whether or not the pollinating species of *Dracula* also visit co-occurring fungi we made extensive collections (>1,000 individuals) at both *Dracula* flowers and co-occurring mushrooms. We have also reared over 1,000 individuals from both mushrooms and flowers, and sampled the background flying insect community with malaise traps (~9,000 individuals). Processing the entomological specimens includes detailed morphological examination, photomicrography (Olympus SZX16), and 'DNA barcoding' of the COI gene (Hebert et al. 2003).

Aspirators. - To connect each individual insect specifically to an individual flower or individual mushroom we employed handheld aspirators (BioQuip Products. Rancho Dominguez, CA.). Collections were made at the flowers of *Dracula* species including *Dracula chiroptera, D. felix, D. lafleurii, D. morleyi, D cf. pubescens, D. sodiroi* (n=74 total), as well as at the fruiting bodies of co-occurring fungi (n= 88). We also collected insects from the flowers of non-mushroom mimicking orchids for comparison. These co-occurring, related species included *Masdevallia nidifica* (24)*, M. ophioglossa* (1)*, M. ximenae* (1)*, Pleurothallis restrepiodes* (1)*,* and *Poroglossum hoeijeri* (1) (n=28 total).

Rearing. - To determine which insects were successfully using mushrooms and/or flowers as substrates for oviposition and larval development we implemented a rearing program from diverse substrates.

In 2010 a pilot study was conducted wherein we collected three species of *Dracula* flowers [*D. lafluerii* (n=2)*, D. morleyi* (n=1)*, D.* cf. *pubescens* (n=1)] and four species of mushrooms [*Filoboletus gracilis* (Mycenaceae) (n=2), *Polyporus craterellus* (Polyporaceae RLC 717) (n=7) and the unidentified collections RLC 719 (n=1) and 720 (n=2)] and incubated them at ambient temperature (15-25 °C) in glass jars (355ml) (n=21) covered with an air-permeable polyester mesh. To maintain humidity, wet cotton balls were placed in each jar. Each jar was monitored daily from March 20 to May 11 and any adult insects were 'harvested' into 95% ethanol.

In 2011 a more comprehensive study was undertaken. A range of substrates were collected and incubated at ambient temperature in plastic tubs (750 ml), most of which contained imported, sterilized, moist hardwood sawdust (from trees felled at the Royal

Botanic Gardens, Kew) and some of which contained moistened cotton balls, which were locally available (n=75 tubs total). Of the substrates collected, 33 were mushrooms, and 42 were flowers, from the following *Dracula* species: *D. felix* (14)*, D. lafleurii* (6)*, D. morleyi* (9)*, D. cf. pubescens* (2)*;* and the non-orchid outgroup genera: *Columnea* (1)*, Costus* (1)*, Heliconia* (2)*, Psammisia* (1)*, Renealmia* (3)*, Stromanthe* (1)*,* and *Tibouchina* (2)*.* Tubs were covered with pantyhose and were moistened as needed (~bidaily). Each tub was monitored daily from January 6 to March 8 and any adult insects were preserved in 70% ethanol.

Malaise traps. - To determine whether these flies are specific to mushrooms and mushroom-mimicking *Dracula* orchids, or simply common in the environment, we surveyed the background flying insect community using malaise traps [n=4; two trapping locations/year for two years (2010 & 2011)]. One of the trapping locations was in the Los Cedros river valley, near the river at 1320m elevation. This site was near the only populations of *D. lafleurii*. The other trapping site was on a ridge top at 1655 m elevation, where *D. felix* is abundant.

Identification

Initial processing of specimens involved sorting to order and family (Brown et al. 2009, 2010) under a portable stereoscope (ESH200 Ken-a-vision, Kansas City, MO) in the field. The next step was gross imaging with a camera-mounted stereoscope (Olympus SZX16) and removal of single legs for DNA extraction. Morphological identification was performed using typical characters including male genitalia for 354 specimens (Grimaldi 1986). DNA barcoding of the COI gene (Hebert et al. 2003) was done to support the morphological work (n=604 individuals) using the following protocol: DNA was

extracted by preparing 0.5-1 mm^3 of fresh insect tissue (one leg) from each specimen with reduced volumes (due to the minute size of the legs) of the recommended recipe for the prepGEM (ZyGEM, New Zealand) insect DNA extraction kit protocol (17.5 ul sterile water, 2 ul 10x buffer, 0.5 ul prepGEM per sample). The tissue and extraction solution assembly was then incubated at 75C° for 15 min then 95C° for 5 minutes (prepGEM protocol).

The COI gene was amplified and sequenced using primers HCO2198 and LCO1490 (Folmer et al. 1994). Each polymerase chain reaction (PCR) vessel contained 2 ul of diluted DNA extract as template along with a 8 ul PCR mix containing 5 ul Jumpstart Readymix (Sigma-Aldrich), 2.2 sterile water, 0.2 ul (10 mM) of each primer (Eurofins Genomics**,** Huntsville, AL) and supplementary $MgCl_2$ (Sigma-Aldrich) (0.4 ul at 25 mM) to maintain a final $MgCl_2$ concentration of 2.5 mM. Reactions were amplified using the following thermal cycle program (Applied Biosystems Veriti™): 1 cycle at 94C° for 1 min.; five cycles of 94C° for 1 min., 45C° for 1.5 min. and 72C° for 1.5 min; 35 cycles of 94C° for 1 min, 50C° for 1.5 min. and 72C° for 1 min. and a single cycle of 72C° at 5 min. (Hebert et al. 2003).

PCR products were visualized on a 1% agarose gel to confirm DNA amplification. Successful PCR reactions were subsequently purified before sequencing by 0.4 volumes of a mixture containing shrimp alkaline phosphatase (0.05 units/mL) and exonuclease I (0.05units/mL) in water and heated for 15 min. at 37C° followed by 15 min. at 85C° (Thermo Scientific protocol). Forward and reverse unidirectional Sanger sequencing was done by Functional Biosciences, Inc. (Madison, WI).

Sequences were edited in Geneious (Biomatters_Ltd. 2013) and OTUs were assigned at the 99% similarity cut-off with Qiime (Caporaso et al. 2010, Edgar 2010). A stringent 99% similarity cut-off was used instead of the commonly accepted 97% cut-off for morphologically problematic organisms like bacteria (Nemergut et al. 2011). The cut-off was determined empirically by coupling morphological comparisons [body size, body and wing coloration, setation, other body structures (e.g., facial carina, arista), and detailed structure of male and female genitalia] with the molecular barcoding we were able to determine what level of genetic variation corresponds to consistent, species-specific morphological differences. In the groups of drosophilids in our study, the most accurate cut-off was 99% similarity. Most of the *Hirtodrosophila* and *Zygothrica* species are referred to by numbers (e.g., "sp. 32"), because these species could not be unambiguously identified as a described species using current monographs (Burla 1956, Grimaldi 1987, 1990b, a). Indeed, most of the species in this study appear to be undescribed species.

Behavioral observations of drosophilid flies

Field observations (19 x 30min. observation periods = 9.5 hrs.) and video recordings (12 x 80 min. = 16hrs.) were made in 2010. Behaviors specifically recorded included: standing, roaming, semaphoring [wing movements that have been implicated in courtship (Grimaldi 1987, Burla 1990)], lapping at the substrate surface with mouthparts, confrontations, and mating.

Analysis

The statistical analyses in this study are limited to the 669 drosophilids that we could identify at least to morphospecies . The first analysis performed was a species

richness estimation to determine the amount of the community diversity we had captured. The number of fly species collected was plotted against the number of collection events (Figure 4.2) (73 samples from *Dracula* flowers, 30 samples from mushrooms). First and second order jackknife, as well as classic, and bias-corrected Chao2 estimates of total species richness were calculated in PC-ORD v.6.14 (McCune and Mefford 2011).

To test hypotheses about the association of visitor communities to the different substrates we used a community dissimilarity matrix approach. For this analysis we included all 105 collection events, but collapsed the data into 17 broad groups: *Dracula* spp. (6 species), mushroom families (9 families), and outgroups (one related, but non-mushroom-mimicking orchid: *Pleurothallis restrepiodes*, and one malaise trap capture). Using Mantel tests [PC-ORD v.6 (McCune and Mefford 2011)] we tested a 17x17 dissimilarity matrix {Bray-Curtis distance [vegan package for R (Oksanen et al. 2013)]} against a series of hypothesis matrices to determine associations between communities. We compared the communities visiting different mushrooms, and the communities visiting different *Dracula* species. We compared the community visiting *Dracula* to the community visiting mushrooms, and the community of visitors to the outgroups (all hypotheses shown in Table 4.2).

Figure 4.4 shows a graphical representation of the overlap between the fly communities collected on mushrooms, orchids and outgroups. The collection from *Dracula sodiroi* was considered an 'outgroup' for the production of this figure due to its atypical *Dracula* phenotype and the results of our community comparisons above which showed the insect collection to be more like the outgroups than like the other *Dracula* spp. (Table 4.2). Figure 4.4 was produced by performing nonmetric multidimensional

scaling (NMS) in PC-ORD (McCune and Mefford 2011) on 72 drosophilid species captured during 105 collection events [30 from mushrooms, 73 from mushroom-mimicking *Dracula* spp. (1 *D. chiroptera*, 41 *D. felix*, 19 *D. lafleurii*, 6 *D. morleyi*, 6 *D. pubescens*), and two from non-mushroom-mimicking orchids, *D. sodiroi* and *Pleurothallis restrepioides*].

We tested for host use preference in the wild collections using replicated goodness-of-fit tests (G-tests) of our observed frequencies against the null hypothesis of random visitation (Sokal and Rohlf 1995, Rohlf 1999). Expected frequencies were calculated based on the number of collection events from each substrate and the assumption of random visitation Low p values reject the null hypothesis (Table 4.1).

From our field observations and our video archives we measured how much time was spent performing each type of behavior. We then divided the amount of time (seconds) by the number of flies that visited during the observation period to estimate the amount of time spent per fly, this was used in subsequent analysis as our measure of duration. We also calculated the proportion of its visit that each fly spent performing each behavior. We analyzed both of these response variables by a 2-way ANOVA with a model that included substrate, behavior, and any interaction between them (SAS 2010).

RESULTS

Fly visitation to *Dracula* does not merely reflect the overall diversity of the mycophilous or anthophilous groups of Drosophilidae; it is far more skewed and selective. Specifically, the visitors are mostly from the *Zygothrica vittatifrons* and *poeyi* species groups; few are from the diverse *dispar, aldrichi*, and *atriangula* groups, and few

species are from the speciose genus *Hirtodrosphila*. Likewise, there were no *Dracula* visitors from the mycophagous genera *Mycodrosophila* or *Paramycodrosophila*, both of which occur in the area. This may be due to the more specialized host relationships of these genera, which seem to be restricted to the undersides (sporulating surface) of fresh, woody polypores (Grimaldi pers. obs.). Other mycophagous drosophilids in the local area but not occurring at *Dracula* include *Leucophenga* and assorted species in the large *Drosophila tripunctata* group.

Dracula ***spp. attract the same fly species as mushrooms***

Collections (976 individuals) from both *Dracula* flowers and co-occurring mushrooms were made up overwhelmingly of flies (Diptera) [89% overall; 35% from *Dracula* flowers (n=73) and 54% from mushrooms (n=30)] with beetles (Coleoptera) being a distant second (7% total) (Figure 4.1). Within Diptera (860 individuals), the most abundant family by far is Drosophilidae (95% overall; 38% from *Dracula* flowers and 57% from mushrooms; Figure 4.2).

Using a combination of morphology and COI sequencing we identified half of the Drosophilidae collected to species (Appendix C). Based on these 432 individuals, representing 73 species from 105 collections, we produced the species accumulation curves shown in Figure 4.3. These curves suggest that we captured and identified 54%-66% of the *Dracula* visitor diversity and 52%-63% of the mushroom visitor diversity, depending on the estimation method (jackknife vs. Chao2). Of the 432 identified flies, 40 either occurred as singletons in the collection, or were collected from a substrate with little replication [*Dracula chiroptera* (1), *D. morleyi* (6), *D.* cf. *pubescens* (6),

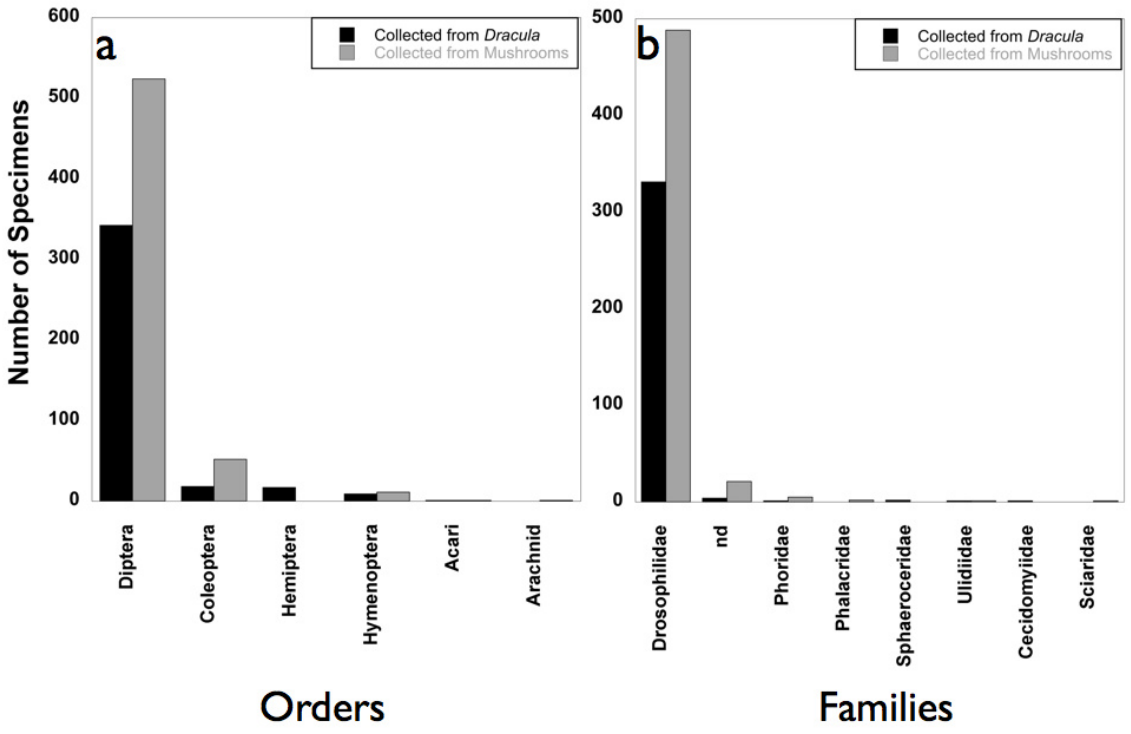

Figure 4.2. Members of Drosophilidae and other dipterans composed the majority of collections from both *Dracula* flowers and co-occurring mushrooms. The ordinal and family level results of our insect collections at mushroom-mimicking *Dracula* flowers and their putative models in a cloud forest in Ecuador [~1000 individuals from 73 flowers (6 species of *Dracula*) and 30 mushrooms (representing 9 different families)].

D. sodiroi (1), control flowers (1)] and therefore were omitted from the summary table and subsequent host use analyses (Table 4.1). However, they are included in the full species list (Appendix C) for completeness.

The 392 individuals representing 47 species identified from *Dracula felix* and *D. lafleurii* and co-occurring mushrooms are summarized in Table 4.1. Our data show considerable overlap in visitors to the orchid species with those to nearby mushrooms. Of the 35 species collected on mushrooms, 24 (69%) also were found on at least one

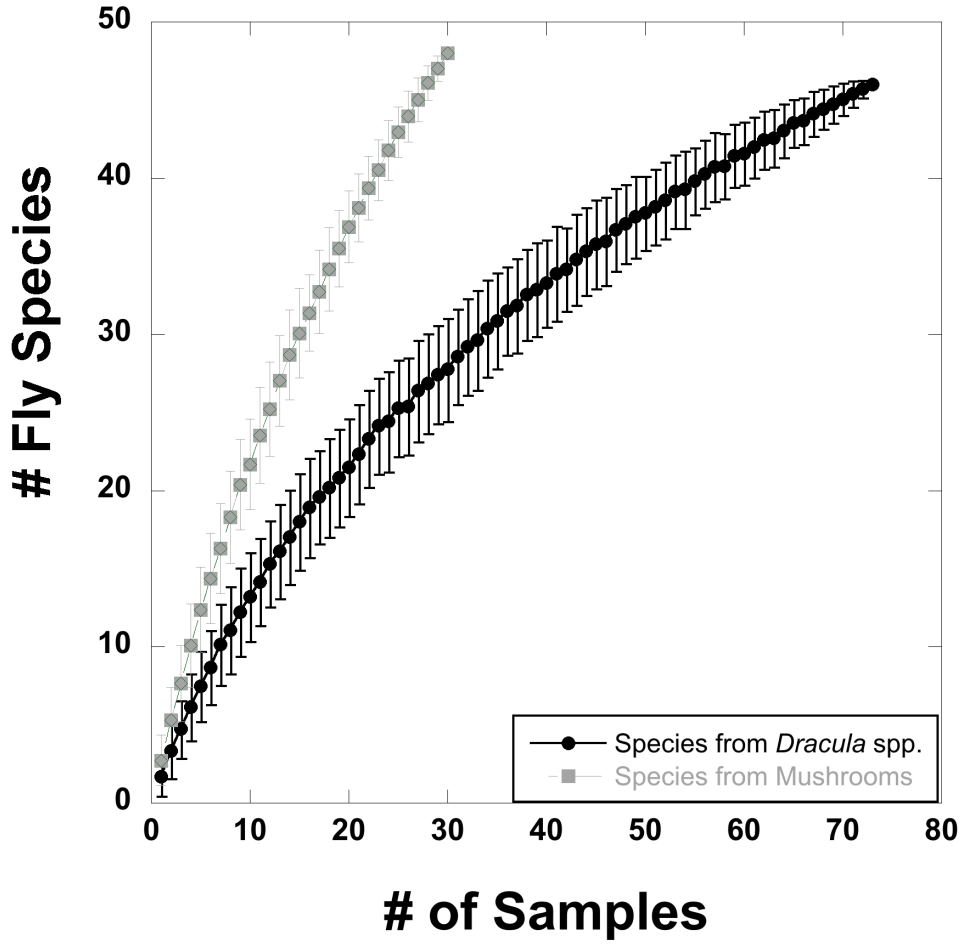

Figure 4.3. Species accumulation curves for drosophilid visitors to *Dracula* spp. and co-occurring mushrooms suggest we have captured over half of the existing diversity. We have identified 46 species from 73 *Dracula* flowers (6 different species). Estimates of the total number of species depended on the test used; 68.7 (1° jackknife), 77.2 (Chao2, bias-corrected), 83.8 (Chao2, classic), and 84.3 (2° jackknife). Comparing our collection numbers to the estimated suggests we have captured from 54.8%-66.7% of the *Dracula* visitor diversity. We have identified 48 species from 30 different mushrooms (representing nine families). Estimates of the total number of species again depended on the test used; 76.0 (1° jackknife), 76.0 (Chao2, bias-corrected), 80.4 [Chao2, classic), and 91.4 (2° jackknife). These numbers suggests that we have captured from 52.7%-63.2% of the mushroom visitor diversity.

of the *Dracula* spp. Of the 27 species collected on *D. felix*, 21 (78%) also were collected from mushrooms, and of the 17 caught on *D. lafleurii* 10 (59%) also were caught on mushrooms. Of the 35 species captured on the two species of *Dracula*, only 9 species (26%) were shared between them.

Table 4.1. Summary of Drosophilid Visitors, Breeders, and Pollinators to Mushrooms and Co-occurring *Dracula felix* and *D. lafleurii* (excluding singletons)

	Fly Species Ranked by abundance in field collections	Collections Expected Ratio: 1.6 : 2.2 : 1.0				Reared Expected Ratio: 5.8 : 1.8 : 1.0				All	Substrate Preference in wild collections Replicated Goodness of Fit		
		Mushrooms (n=30)	**D. felix (n=41)**	**D. lafleurii (n=19)**	**Total**	**Mushrooms (n=46)**	**D. felix (n=14)**	**D. lafleurii (n=8)**	**Total**	**TOTALS**	**G**	**df**	**p**
1	***Zygothrica 41***	**32**	**3**		**35**	**98**			**98**	**133**	**54.55**	**2**	**<0.0001**
2	*Zygothrica 6*		17[$]	16	33				0	33	30.79	2	<0.0001
3	*Zygothrica 16*		29[$]	1	30				0	30	39.94	2	<0.0001
4	*Zygothrica 10*			29	29				0	29	90.21	2	<0.0001
5	***Hirtodrosophila 7***	**25[$]**	**1**		**26**	**27**			**27**	**53**	**48.03**	**2**	**<0.0001**
6	***Zygothrica 3***	**2**	**22[$]**		**24**				**0**	**24**	**25.22**	**2**	**<0.0001**
7	***Zygothrica 29***	**15**	**5**		**20**				**0**	**20**	**27.08**	**2**	**<0.0001**
8	***Zygothrica 9***	**17**	**1**	**1**	**19**	**5**			**5**	**24**	**33.25**	**2**	**<0.0001**
9	***Zygothrica 20***	**2**	**14[$]**	**1**	**17**	**9**			**9**	**26**	**15.75**	**2**	**0.0004**
10	***Zygothrica 8***	**11**		**3[$]**	**14**				**0**	**14**	**20.46**	**2**	**<0.0001**
11	***Hirtodrosophila 6***	**9**	**4**		**13**	**4**			**4**	**17**	**15.7**	**2**	**0.0004**
12	***Zygothrica prensiseta***	**4[$]**	**3**	**2**	**9**				**0**	**9**	**1.49**	**2**	**0.4742**
13	*Zygothrica 48*	9			9	4			4	13	19.78	2	<0.0001
14	*Zygothrica 12*	8[$]			8	2			2	10			nd
15	***Zygothrica 26***	**6[$]**		**1**	**7**				**0**	**7**			nd
16	***Hirtodrosophila 2***	**3**	**2**		**5**				**0**	**5**			nd
17	***Hirtodrosophila 9***	**3**	**2**		**5**	**6**			**6**	**11**			nd

18	***Zygothrica 2***	**2**	**2**	**1**	**5**	**15**		**15**	**20**	nd
19	*Zygothrica 4*			5	5			0	5	nd
20	*Zygothrica 46*	5			5	29		29	34	nd
21	***Zygothrica 5***	**1**	**1**	**3**	**5**			**0**	**5**	nd
22	*Drosophila 1*	4			4			0	4	nd
23	*Drosophila new*	4			4			0	4	nd
24	***Hirtodrosophila 8***	**3**	**1**		**4**	**6**		**6**	**10**	nd
25	***Zygothrica 21***	**1**	**2**	**1**	**4**			**0**	**4**	nd
26	***Zygothrica 57***	**1**		**3**	**4**			**0**	**4**	nd
27	***Hirtodrosophila 1***	**2**	**1**		**3**			**0**	**3**	nd
28	*Hirtodrosophila 3*	3			3			0	3	nd
29	*Hirtodrosophila 10*	3			3			0	3	nd
30	*Zygothrica 18*		2	1	3			0	3	nd
31	*Zygothrica 25*			3	3			0	3	nd
32	***Zygothrica 30****	**3**			**3**			**0**	**3**	nd
33	*Zygothrica 32*	3			3			0	3	nd
34	***Zygothrica 49***	**2**	**1**		**3**			**0**	**3**	nd
35	***Zygothrica 56***	**2**[$]	**1**[$]		**3**			**0**	**3**	nd
36	*Laccodrosophila 3*		2		2			0	2	nd
37	*Zygothrica 1*		2		2			0	2	nd
38	*Zygothrica 13*	2			2			0	2	nd
39	***Zygothrica 23***	**1**	**1**		**2**			**0**	**2**	nd
40	*Zygothrica 24*		2		2			0	2	nd
41	***Zygothrica 31***	**1**	**1**		**2**			**0**	**2**	nd
42	***Zygothrica 33***	**1**		**1**[$]	**2**			**0**	**2**	nd
43	*Zygothrica caputrichia*	2			2			0	2	nd
44	*Cladochaeta A*			1	1		2	1	3	4	nd
45	***Zygothrica 36***		**1**		**1**	**2**			**2**	**3**	nd
46	*Zygothrica 47*	1			1	2			2	3	nd
47	***Zygothrica 53***		**1**		**1**	**1**			**1**	**2**	nd

TOTALS	**195**	**124**	**73**	**392**	**213**	**4**	**1**	**218**	**610**			
Total observed ratios	**2.7**	**1.7**	**1**		**213**	**4**	**1**			**Substrate preference in wild collections** Replicated Goodness of Fit		
Expected ratios	**1.6**	**2.2**	**1**		**5.8**	**1.8**	**1**			**G**	**df**	**p**
Pooled totals first 13 rows	**126**	**99**	**53**				**Total G**			**422.25**	**26**	**<0.0001**
Pooled observed first 13 rows	**2.4**	**1.9**	**1**				**Pooled G**			**22.85**	**2**	**<0.0001**
							Heterogeneity G			399.40	24	<0.0001

These data represent all of the identified flies that we either aspirated *in situ* (collections) or reared from collected substrates. We tested for visitor preference in the wild collections using replicated goodness of fit (G-tests) tests of our observed frequencies against the null hypothesis of random visitation. Low p values reject the null hypothesis. Expected frequencies were generated based on the number of times that collections were made at each substrate. G-tests are only recommended for expected frequencies of five or more; nd (no data) indicates species where we had too few individuals to perform the G-tests. Statistics at the bottom show that overall our observed frequencies are different from expected (Total G), that the pooled data (Pooled G) are different from the expected ratios, and that there are different ratios across each of the tests (Heterogeneity G) which disallows hypothesis testing with pooled data. Notably all species tested showed a strong preference [except *Z. prensiseta* (row12)] despite being found on more than one substrate. No species that hatched out of mushrooms also hatched out of *Dracula* flowers and vice versa, indicating very strong hatching substrate preference in all cases. **Bold** font indicates 'shared' species that are found on mushrooms and at least one of the *Dracula* spp. *Z. 30 (row 32) is **bolded** indicating that it is a shared species, however it is shared between mushrooms and one collection from a *D. pubescens* (listed in Sup. Table1).'$' indicates species and substrates that we have associated with orchid pollinia.

The results of our malaise trap survey show that these flies are specific to mushrooms and mushroom-mimicking *Dracula* orchids. We collected 8,821 individuals from malaise traps, with the majority (85%) of them being members of Diptera. However, within Diptera (7,491 individuals) only 0.5% of individuals were members of Drosophilidae. The most abundant identified dipteran families were Sciaridae and Phoridae at ~24% each.

The communities that visit **Dracula** *are similar to those on mushrooms*

With the establishment of shared species, the next hypothesis tested was: to what degree are the communities visiting each substrate associated (Table 4.2)? Communities from mushroom families were significantly associated with other mushroom families ($p=0.0260$), but communities from *Dracula* species were not necessarily associated with communities from other *Dracula* species ($p=0.8248$). This was true whether we looked at all *Dracula* species or just at the two most common species, *D. felix* and *D. lafleurii* ($p=0.1151$). Despite this heterogeneity within the *Dracula* group itself, there was a positive association between the *Dracula* communities and the mushroom family communities ($p=0.0731$). This association strengthened when the non-mushroom-like *D. sodiroi* was omitted from the *Dracula* group ($p=0.0080$). The collection from *D. sodiroi* was a single individual of *Zapriothrica* (the only member of that genus in our entire collection), making it an outlier.

Figure 4.4 shows a graphical representation of the overlap between the fly communities collected on mushrooms, orchids and outgroups. The relevant statistical tests are the Mantel tests reported above and Figure 4.4 is solely provided as a visual aid. It is notable that only 5 visitor communities (of 30) from mushrooms fall outside of the

convex hulls for *Dracula*, and only 15 (of 73) *Dracula* communities fall outside of the convex hulls for mushrooms.

Table 4.2. *Dracula* spp. Share Visitor Communities with Co-occurring Mushrooms, but Not with Out-groups

Group	Group	Std. Mantel	Z (observed)	Z (random)	p
Mushrooms	**Mushrooms**	**-0.3511**	**3.01E+01**	**3.26E-01**	**0.0260**
Dracula (all spp)	*Dracula* (all spp)	0.1258	1.41E+01	1.63E-01	0.8248
Dracula (all spp)	Mushrooms	-0.3088	9.31E+01	9.52E-01	0.0731
***Dracula* (all *spp* - *sodiroi*)**	**Mushrooms**	**-0.4417**	**7.91E+01**	**8.25E-01**	**0.0080**
D. felix* & *D.lafluerii	**Mushrooms**	**-0.5104**	**4.57E+01**	**4.98E-01**	**0.0030**
D. felix	*D. lafleurii*	-0.1312	7.20E-01	9.07E-01	0.1151
D. felix	**Mushrooms**	**-0.4714**	**3.71E+01**	**4.08E-01**	**0.0110**
D. lafleurii	**Mushrooms**	**-0.3663**	**3.79E+01**	**4.08E-01**	**0.0200**
Mushrooms	Outgroups	-0.1521	4.86E+01	4.98E-01	0.1892
***Dracula* (all *spp*)**	**Outgroups**	**0.3511**	**9.33E+01**	**9.06E-01**	**0.0230**
Dracula (all *spp* - *sodiroi*)	Outgroups	0.1346	7.73E+01	7.62E-01	0.2232

Results of Mantel tests comparing a 17x17 dissimilarity matrix (Bray-Curtis distance) of drosophilid visitors to *Dracula* spp. (6), mushroom families (9), and outgroups (1 related, but non-mushroom-mimicking orchid: *Pleurothallis restrepiodes*, and 1 malaise trap capture). Monte Carlo tests included 999 randomized runs with the null hypothesis that there is no relationship between matrices; low p value rejects the null hypothesis. An observed Z greater than the average Z from randomized runs indicates a positive association between matrices, which is the case in all comparisons that show significant relationships. **Bold** font indicates significant positive associations.

The same individuals move between Dracula *spp. and mushrooms*

Individual flies move between flowers and mushrooms (unpub. and in prep.). We have photographed and collected flies on mushrooms that had orchid pollinia stuck to their thoraces, including one *Hirtodrosophila sp.7*, two *Zygothrica sp.12*, one *Zygothrica sp. 26*, and one *Zygothrica prensiseta* from mushrooms (all *Armillaria* sp.

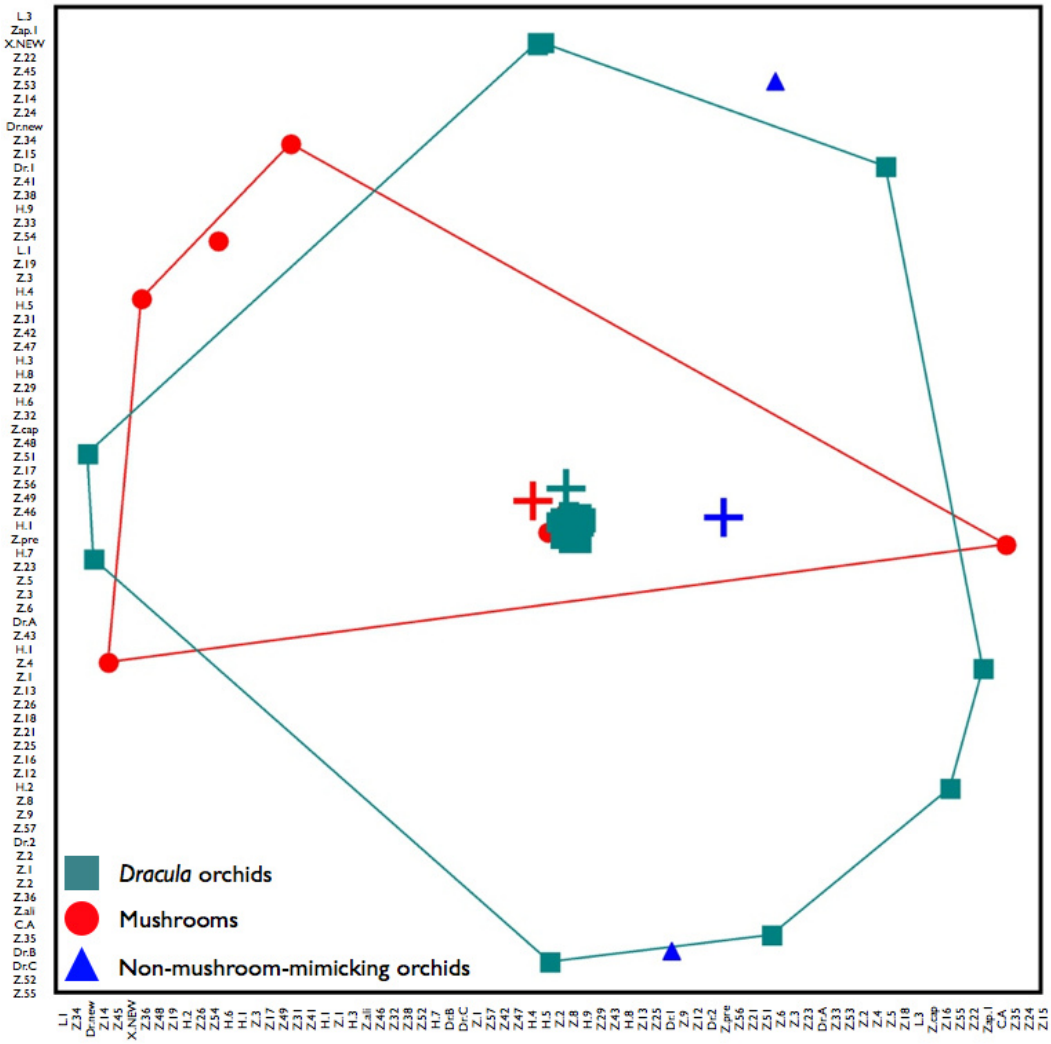

Figure 4.4. Overlapping visitor guilds between *Dracula* spp. and co-occurring mushrooms. Graphical representation of the overlap in the communities visiting these substrates. Two dimensional image produced by nonmetric multidimensional scaling (NMS) of 72 drosophilid species captured over 105 collection events [30 from mushrooms, 73 from mushroom-mimicking *Dracula* spp. (1 *D. chiroptera*, 41 *D. felix*, 19 *D. lafleurii*, 6 *D. morleyi*, 6 *D. pubescens*), and two from non-mushroom-mimicking orchids, *D. sodoroi* and *Pleurothallis restrepioides*]. **'+'**s mark centroids for each group which are also bounded by convex hulls. Notably only 5 visitor communities on mushrooms fall outside of the convex hulls for *Dracula*, and 15 *Dracula* communities fall outside of the convex hulls for mushrooms (the uppermost cluster of squares represent 8 collections). The clusters in the center represent communities from 58 *Dracula* flowers and 25 mushrooms. While the centroid marker for the two communities from non-mushroom-mimicking orchids lands quite close to the other centroid markers, the two points that it is based on fall outside of the convex hulls of both other groups. X and Y axes are populated with the 72 fly species. (C.= *Cladochaeta*, Dr.=*Drosophila*, H.=*Hirtodrosophila*, L.=*Laccodrosophila*, X.=novo genus, Z.=*Zygothrica*, Zap.=*Zapriothrica*. Z.ali=*Z.aliucapa*, Z.cap= *Z.caputrichia*, Z.pre=*Z.prensiseta*).

Physalacriaceae) (rows 5, 14, 15 and 12 respectively in Table 4.1). Also an individual similar to *Zygothrica* sp.*10* was collected with pollinia on the same substrate, but then omitted from Table 4.1 due to uncertain identity. Our collection includes 7 other unidentified drosophilid flies from *Armillaria* and one from a *Pleurotus sp.* (Pleurotaceae RLC 668) all carrying pollinia. The central panel in Figure 4.1 shows an unidentified *Zygothrica* species carrying orchid pollinia while visiting a *Polyporus craterellus* (Polyporaceae RLC 717). Field notes also record an unidentified drosophilid carrying pollinia while on an unidentified mushroom (pers. obs. Policha, Jan. 6, 2012). These pollinia are difficult to unambiguously identify, but are the same size, shape and color as pollinia from *Dracula* spp.

Drosophilid visitor's host-use between substrates

Overall the observed frequencies of collected flies differed significantly from random (Total $G=422.25$, $df=26$, $p<0.0001$), suggesting preference. The pooled data are also different from the expected ratios (Pooled $G=22.85$, $df=2$, $p<0.0001$), however there are differences across each of the 13 tests (Heterogeneity $G=399.40$, $df=24$, $p<0.0001$), disallowing hypothesis-testing with pooled data (Sokal and Rohlf 1995). Notably all species tested showed a strong preference, despite being found on more than one substrate, with the exception of *Zygothrica prensiseta* (Table 4.1, row 12), which was found everywhere. Of the nine shared species we tested, six preferred mushrooms (Table 4.1, rows 1, 5, 7, 8, 10 & 11), two preferred *Dracula felix* (Table 4.1, rows 6 & 9), and *Zygothrica prensiseta* was a generalist. Of the 13 most abundant species, only *Zygothrica* sp. *48* was found solely on mushrooms (Table 4.1, row 13). Of the three species found only on *Dracula* flowers, *Zygothrica* sp.*16* appears to be a *D. felix* specialist (Table 4.1,

row 3), *Zygothrica* sp. *10* appears to be a *D. lafleurii* specialist (only found on that substrate Table 1, row 4), and *Zygothrica* sp. *6* was found in equal numbers on both orchid species (Table 1, row 2). These drosophilids show a range of preference, from being found on a single substrate (Table 4.1, rows 4 & 13), to being complete generalists like *Zygothrica prensiseta.*

***Dipteran visitors to mushrooms*, Dracula felix *and* D. lafleurii, display similar patterns of behavior across the three substrates**

Visitors showed differences in time spent performing each behavior across the three substrates, (whole model $F_{17,174}$=29.93, p<0.0001, Figure 4.5). Flies also had longer visits to *D. lafleurii* than to *D. felix*, with time spent on mushrooms being intermediate (not significantly different from either *Dracula* species) (F=3.16, df=2, p=0.0448). On all three substrates flies spent the most time standing still. Time spent walking, probing, and semaphoring was significantly greater than time spent either mating or fighting (F=76.35, df=5, p<0.0001). Despite the overall similarity in behavioral patterns across the three substrates, there was a significant substrate x behavior interaction (F=3.04, df=10, p=0.0014). Generally, the flies were more active on *D. lafleurii* as evidenced by slightly less time spent standing. The time spent extending proboscises on *D. lafleurii* was significantly higher than on *D. felix*, with proboscis extension on mushrooms being of intermediate duration.

In terms of the proportion of each fly's time budget, there were also significant differences (whole model $F_{17,174}$=102.71 p<0.0001), but similar patterns. Again, standing occupied more time than any other activity, while mating and fighting took up the least (F=255.09, df=5, p<0.0001). The proportion data show significantly more activity by

flies on *D. lafleurii* as evidenced by a smaller proportion of time standing (Substrate x behavior F=6.77, df=10, p<0.0001), although the differences in other behaviors are not significant.

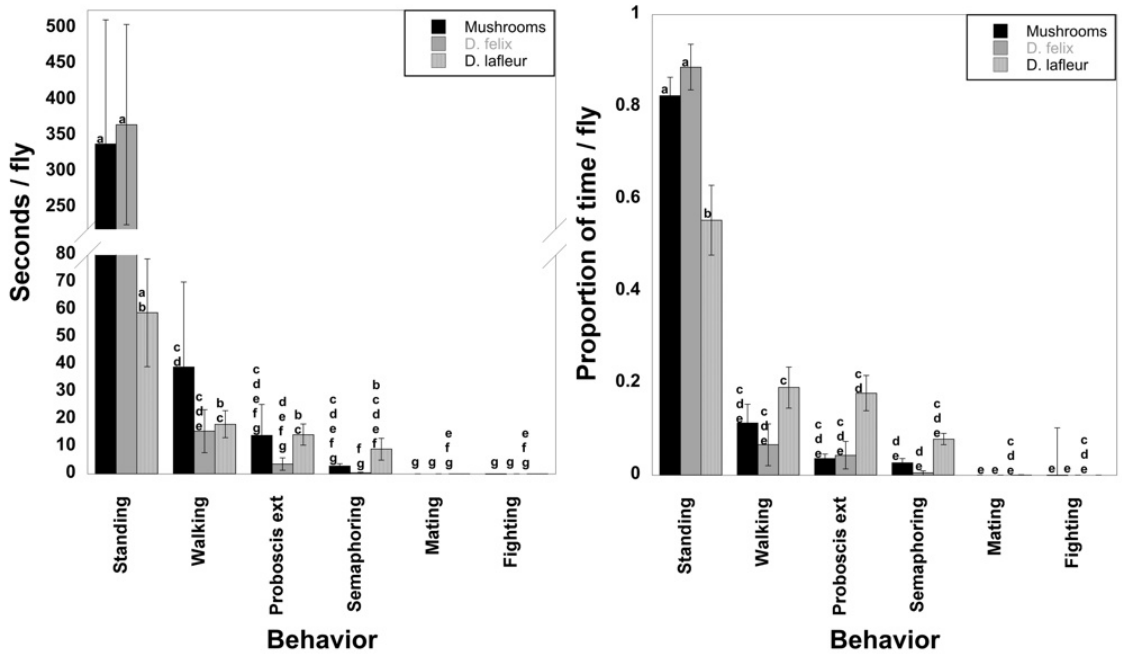

Figure 4.5. Dipteran visitors to mushrooms, *Dracula felix* and *D. lafleurii*, display similar patterns of behavior across the three substrates. We analyzed both the absolute amount of time spent per fly as well as the proportion of the total time spent by each fly. Visitors showed differences in time spent in each behavior across the three substrates, whole model $F_{17,174}$=29.93, p<0.0001 (data was log transformed prior to analysis to normalize residuals although raw data is presented in the figure); flies spent more time on *D. lafleurii* than on *D. felix*, with time spent on mushrooms being intermediate (not significantly different from either *Dracula* species) (F=3.16, df=2, p=0.0448). On all three substrates flies spent the most time standing still. Time spent walking, probing, and semaphoring was significantly more than either mating or fighting (F=76.35, df=5, p<0.0001). Despite the overall similarity in behavioral patterns across the three substrates, there was a significant substrate x behavior interaction (F=3.04, df=10, p=0.0014). These details are shown above, with levels not connected by same letter being significantly different (Tukey's HSD). Generally the flies were more active on *D. lafleurii* as evidenced by slightly less time standing. Time spent extending proboscises on *D. lafleurii* was significantly higher than on *D. felix*. In terms of proportion of time budget for each fly, we again saw significant differences (whole model $F_{17,174}$=102.71 p<0.0001), but similar patterns. Again standing took up more time than any other activity, while mating and fighting took up the least (F=255.09, df=5, p<0.0001). The proportion data show significantly more activity by flies on *D. lafleurii* as evidenced by a smaller proportion of time standing (Substrate x behavior F=6.77, df=10, p<0.0001), although the differences in other behaviors are not significant [levels not connected by same letter are significantly different (Tukey's HSD)]. N=*D. felix* 12 observation periods, *D. lafleurii* 9, and mushrooms 17.

Drosophilids are breeding in mushrooms and Dracula *orchids*

Ninety seven percent of the 1,288 insects that we reared out of mushrooms and *Dracula* flowers were flies (Diptera) (Figure 4.6). At the family level, of the 1,250 flies that hatched, 52% were from the family Drosophilidae. The Phoridae and Cecidomyiidae were also well represented from mushrooms (13% and 12% respectively). Two major findings stand out from this study: 1. 95% of all flies were reared from mushrooms; and 2. No species that hatched out of mushrooms also hatched out of *Dracula* flowers and vice versa (Table 4.1).

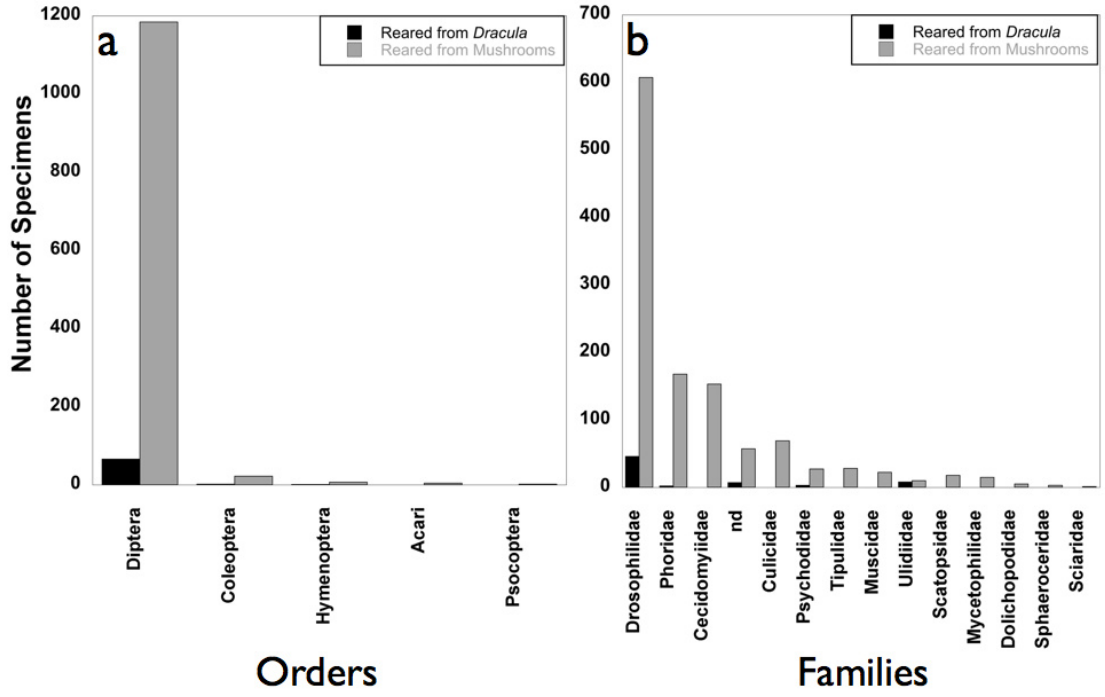

Figure 4.6. Members of Drosophilidae and other dipterans composed the majority of reared specimens from both *Dracula* flowers and co-occurring mushrooms. The ordinal (a) and family (b) level results of our insect rearing from *Dracula* flowers and their mushroom models in a cloud forest in Ecuador (>1200 individuals from 35 flowers and 45 mushrooms). The mushrooms were a much more productive substrate and at the species level that was no overlap in species reared from *Dracula* and species reared from mushrooms (See Table 4.1).

See Appendix C for a full list of identified Drosophilidae. Insect collections are deposited at the Sección de Invertebrados del Instituto de Ciencias Biológicas, Escuela Politécnica Nacional, Quito, Ecuador, including holotypes. Duplicates, including some paratypes, are deposited at the American Museum of Natural History, New York, NY. See Supplemental Figure S4.3 (Appendix A) for photos of some of the specimens.

DISCUSSION

Zygothrica is by far the most significant group of *Dracula* visitors (197 individuals representing 37 species, Appendix C). This is a very large genus of Drosophilidae, with 126 described species (111 of them Neotropical). Revisions of large portions of the genus are still needed (Burla 1956, Grimaldi 1987, 1990b, a), particularly striped species of the *vittatifrons* and *poeyi* species groups, which are also the most abundant and diverse groups of *Zygothrica* visiting *Dracula*. In the New World the genus occurs from southern Mexico to Bolivia and northern Argentina, which overlaps the range of *Dracula* [from southern Mexico to northern Peru (Luer 1993)]. It should be noted that the association of *Zygothrica* with *Dracula* is probably widespread, and not restricted to the area in Ecuador where these studies have been done. There are unidentified *Zygothrica* specimens in the AMNH collected from *Dracula* flowers in Colombia and Panama.

Zygothrica individuals were also frequently caught on mushrooms in this study (142 individuals representing 33 species, Appendix C). *Zygothrica* in general are well known from fleshy, white sporocarps, where they can congregate by the thousands on top of and under the pilei, actively displaying, fighting, and grazing (Grimaldi pers. obs.).

Zygothrica are almost always dark-bodied or bold-patterned flies that contrast against the light background of the mushrooms where they congregate. They commonly have stripes on the upper portion of the thorax and the abdomen; sometimes with a dark apical spot or pattern on the wings. Species with wing patterns wave and flick the wings, which clearly are used in signaling for mating, as probably are the body patterns. Males of larger species, particularly the broad-headed species, are often aggressive and territorial toward other flies.

Within *Zygothrica* we see a variety of patterns in host use. There are species that visit both *Dracula* spp. and mushrooms indiscriminately (e.g., Z. *prensiseta*), and species that only visit mushrooms (e.g., *Z.* sp.*48*), only visit *Dracula* spp. (e.g., *Z.* sp.*6 & Z.* sp.*16*), or that even visit only one species of *Dracula* (e.g., *Z.* sp.*10*). Most species for which we had sufficient numbers to analyze statistically seemed to primarily use one or the other substrate (*Dracula* or mushrooms), but could be found at some frequency on the others.

The other genus where we see considerable overlap in visitation between *Dracula* spp. and mushrooms is *Hirtodrosophila*, a more cosmopolitan genus with >150 species (39 described species in the New World, 8 of these in North America). Old World species are fairly well described, but dozens of the Neotropical species are undescribed. Major references for the New World species include Burla (1956) (largely just species from southeast Brazil) and Vilela and Bächli (2004) (a treatment of poorly described types). This genus is probably paraphyletic with respect to the other mycophagous, well-defined genera in the *Zygothrica*-genus group, which includes *Mycodrosophila*, *Paramycodrosophila*, as well as *Zygothrica* (pers. comm. Grimaldi). Species are

generally significantly smaller than in *Zygothrica*, and commonly light colored, rarely with bold patterning. Based on the extensive collections of these flies made by Grimaldi, *Hirtodrosophila* has a broader fungal preference than *Zygothrica*, and visit pliant/moist polypores, Agaricomycetidae sporocarps, *Auricularia,* etc. All of the ten species that we collected in this study were found on mushrooms [Agaricaceae (*Agaricus*), Cortinariaceae (*Gymnopilus*), Marasmiaceae (*Marasmius*) Mycenaceae (*Filoboletus*, *Mycena*), Physalacriaceae (*Armillaria*), Pluteaceae (*Pluteus*), Polyporaceae (*Rigidiporus*), Tricholomataceae (*Collybia*, *Dictyopanus*)], six of which were also collected on *Dracula* spp. The four species that we also reared from mushrooms were a subset of those species that we had collected on *Dracula* (Appendix C). In the two species where we had enough individuals to analyze substrate use (*H.* sp. *6 & H.* sp. *7*), both were collected more than expected at mushrooms, suggesting that they visit *Dracula* incidentally. However we did capture one specimen of *H.* sp.*7* that was carrying orchid pollinia (Table 4.1), so their visitation may be important for fitness in the orchids.

The genus *Laccodrosophila* is one of the few true, specialized flower-breeding groups of Drosophilidae that visited or bred in *Dracula* flowers. Interestingly, they were not very common (12 specimens of 2 different species, to 9 flowers) and were only associated with *D. felix*, *D. morleyi* and *D.* cf. *pubescens*. *Laccodrosophila* are very distinctive, robust drosophilids, most species of which have an oviscapt with large apical "teeth," which telescopes into an ovipositor used for inserting an egg into the ovules of flowers, where the larvae develop. Known hosts (published and unpublished) include the following: *L. takadai* on *Datura* (Solanaceae) flowers in Ecuador (Wheeler 1968); *Laccodrosophila* spp. on *Scaphosepalum* orchids in Reserva Los Cedros, Ecuador (coll.

Endara, Hanneman, Huggins [in AMNH collection]); and *Laccodrosophila* sp. on *Symbolanthus pulcherrimus* (Gentianaceae) and *Pleurothallis ruscifolia* (Orchidaceae) in Costa Rica (unpubl., AMNH Collection).

Laccodrosophila's sister genus, *Zapriothrica*, is usually more common although only one specimen was collected in our study. Species of *Zapriothrica* have been taken on flowers of *Passiflora* (Passifloraceae) in Colombia and Venezuela (Wheeler 1968, Casañas‐Arango et al. 1996, unpubl. [AMNH collections]), *Datura* in Colombia and Ecuador (Wheeler 1956, Wheeler 1959, unpubl. [AMNH collection]), and reared from *Fuchsia* (Onagraceae) in Colombia (unpubl., AMNH coll.). In the current study, only one collection was made from a *Dracula sodiroi* flower (at El Pahuma Orchid Reserve) and the single individual is the only member of *Zapriothrica* in our entire collection. Inclusion here is for the sake of completeness, although the sample size makes firm conclusions impossible and is, in part, why this collection is treated as an outlier in the analysis.

Our investigations into visitor behavior and resource use have been complicated by the impossibility of identifying individual small flies to species in the field. Our behavioral observations then are necessarily a conglomerate of the activity of the ~68 species we know to visit mushrooms, *Dracula felix*, *D. lafleurii*, or some combination of substrates. We do document clear differences in behavior amongst substrates (Figure 4.5), but mostly in terms of the ratio of activity to quiescence. Individual flies tend to be more active on flowers of *D. lafleurii* relative to the other hosts. This may be due to the sheer volume of visitors. When these flowers are maximally attractive, 50-100 individuals visiting per hour is not uncommon (pers. obs., Policha et. al. in prep). This is

consistent with density-dependent behavior displayed by Zygothrica on mushrooms as well. In sparse aggregations of Zygothrica on mushrooms individual flies are not nearly as active (semaphoring, fighting, mating) as when they are in large swarms (Grimaldi pers. obs.).

What are all these flies doing in the flowers? In terms of resource utilization, even when the flies are just standing within the calyx of the *Dracula* flowers, or on the underside of mushroom caps, they are sheltered from the rain (~3,000 mm/yr at our study site). However, the apparent crowding at *Dracula* flowers suggests that shelter may be secondary compared with other functions such as rendezvous sites or feeding. Flies are known to taste with both their proboscis and their feet (Dethier 1976, Barth 1985). We also know that the surfaces of both the *Dracula* flowers and the mushrooms are hosts to yeasts, some of which are also recovered from the gut contents of visiting flies (McAlpine 2013). These two facts support the idea that the roaming and proboscis extension activities may be associated with yeast grazing by the flies. Yeasts are a known food source for many species of Drosophilidae (Starmer 1981). The role of mushrooms as rendezvous sites by flies in these groups is well documented (Parsons 1977, Burla 1990) and the wing-flicking behavior has been suggested to play a role in courtship (Parsons 1977, Burla 1990). These observations, combined with the observed confrontations and matings suggests that the visitors in this study may be utilizing both flower and mushroom resources to obtain mates.

The rearing study yielded more unambiguous results than the field observations, because we could take flies raised from known substrates and identify them with microscopy and DNA barcoding. The most obvious result was that there was no overlap

in species emerging from mushrooms with those emerging from *Dracula* (Table 4.1 and Appendix C). Also of note is the fact that the very few species that we did rear out of *Dracula* flowers were either absent (*Diathoneura*), exceeding rare (*Cladochaeta* sp. *A*), or uncommon (*Laccodrosophila* sp. *3*) in our field collections. This may be an artifact of undersampling (Figure 4.2), or temporal vagaries. Unsurprisingly we reared from mushrooms 43 individuals representing four different species of *Hirtodrosophila*, a genus of mushroom-associated flies. As noted above, all four species also were caught visiting *Dracula* flowers in the field. Again, the best-represented group involved members of the genus *Zygothrica* with eleven species and 170 individuals identified. All of the *Hirtodrosophila* and *Zygothrica* flies emerged only from fungi, and most of these represent new breeding site records. Indeed, there are actually very few records of *Zygothrica* bred from fungi. Breeding site records were reviewed by Grimaldi (1987), with new records provided by dos Santos and Vilela (2005). In total, 17 species of *Zygothrica* have been bred from Acanthaceae (*Aphelandra*), Costaceae (*Calathea*, *Costus*, *Dimerocostus*), Lamiaceae (*Salvia*), Solanaceae (*Brunfelsia*, *Cestrum*, *Sessea*), Passifloraceae (*Passiflora*), and Zingerbaceae (*Hedychium*).

Our data also are interesting for the anthophilous Drosophilidae that were either rare or entirely absent from *Dracula*. These conspicuous absences suggest that *Dracula* is not simply exploiting known flower-breeding insects, despite the diverse flower-breeding species that have evolved in various groups of Drosophilidae (Frota-Pessoa 1952, Heed 1968, Carson and Hartt 1971, Montgomery 1975, Okada 1975, Carson and Okada 1980, Okada and Carson 1980, Brncic 1983). The Neotropical genera *Palmomyia* and *Palmophila* aggregate at inflorescences of palms (Arecaceae) (Grimaldi et al. 2003).

Species in the *Drosophila flavopilosa* group are known almost entirely from flowers of *Cestrum* (Solanaceae) (Wheeler et al. 1962, Brncic 1966, dos Santos and Vilela 2005) and host specialization may explain their absence from *Dracula*. Less specialized flower breeders include the *Drosophila tripunctata* species group (Pipkin et al. 1966, Heed 1968), and the related *Drosophila dreyfusi*, *D. peruviana*, and especially the *D. bromeliae* species groups (Grimaldi et al. 2014). Another unrepresented group in this study is the *Drosophila onycophora* species group (Vilela and Bächli 1990, Figuero and Rafael 2011, Figuero et al. 2012), which includes 19 species confined to the Andean region. Species in this group have been collected or bred from several genera in the Asteraceae (*Montanoa*, *Chrysanthemum*, and *Espeletia*) as well as *Bomarea* (Alstroemeriaceae) and *Cleome* (Cleomaceae) (Hunter 1979, Hunter 1988). none of the six Neotropical species in the *Drosophila* subgenus *Phloridosa* were observed at *Dracula* despite these taxa being known from flowers of *Brugmansia* (Solanaceae) and *Ipomoea* (Convolvulaceae).

Only two specimens in the *D. bromeliae* group were captured at *Dracula* in the study by Endara et al. (2010), and none in the present study (which was done at the same site and amassed many more samples). Very rare visitors to *Dracula* include *Zapriothrica* (discussed above), *Diathoneura* (8 reared specimens, see Appendix C), and *Cladochaeta* (4 specimens, see Table 4.1). Both of the latter genera are exclusively New World in distribution, and with the exception of five Nearctic species of *Cladochaeta*, are entirely Neotropical. There are 38 described species of *Diathoneura* (Vilela and Bächli 1990) and 119 species in *Cladochaeta* (Grimaldi and Nguyen 1999), with hundreds of species that remain undescribed. While the larvae of some *Cladochaeta* are parasites of spittle bug nymphs, some (and probably most) Neotropical species breed in flowers (Grimaldi and

Nguyen 1999). *Diathoneura* spp. were reared in Panama by Pipkin et al. (1966) from *Dimerocostus* (Costaceae), *Heliconia* spp. (Heliconiaceae), *Centropogon* (Campanulaceae), *Helianthus (*Compositae), and *Eichhornia crassipes* (Pontederiaceae). *Diathoneura tessellata* has been reared from *Anaxagorea crassipetala* (Annonaceae) in Costa Rica (Collier and Armstrong 2009). The only published host record of *Cladochaeta* on flowers is from a *Psychotria* (Rubiaceae) in Costa Rica (Grimaldi and Nguyen 1999).

We have shown that *Dracula* spp. share visitors with co-occurring fungi, that there is significant overlap in the communities of visitors coming to both substrates and that the behavior patterns of the visitors are similar across hosts. In addition to accumulating new breeding site records for *Zygothrica*, our data support the brood-site mimicry hypothesis. Flies that otherwise breed in mushrooms are spending time on *Dracula* flowers, where they can move pollinia, but do not breed. These flies are still getting rewards from their visitation such as a mating site, shelter, and possibly grazing on yeasts, so the deception does not lead to a total loss of fitness. Thus, the relationship between *Dracula* orchids and mushrooms falls somewhere along the continuum between Batesian (deception) and Müllerian (convergence) mimicry. Our results also suggest a bi-modal attraction strategy by the flowers, with over half of their visitors belonging to fungal associated taxa. The remaining species appear to be specialists, either visiting exclusively one *Dracula* species or visiting more than one *Dracula* species, but not mushrooms. This apparent specialization may be due to under sampling (Figure 4.2), or alternatively the flies found only on *Dracula* may be obtaining enough of a reward to make this relationship stable.

CHAPTER V

CONCLUSION

"The more I study nature, the more I become impressed, with ever-increasing force, with the conclusion that the contrivances and beautiful adaptations slowly acquired, through each part occasionally varying in a slight degree, but in many ways, transcend in an incomparable degree the contrivances and adaptations which the most fertile imagination of the most imaginative man could suggest with unlimited time at his disposal."

- Charles Darwin
'On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing' (1862).

During the course of this study we have learned a great deal about the pollination biology of *Dracula* orchids. We used novel 3-D printed flowers to decouple the olfactory and visual components of the signaling motif in the orchids. We have revealed aspects of the natural history of dozens of fly species unknown to science, and documented new breeding-site records for the genus *Zygothrica*. We also identified hundreds of cloud forest mushrooms that may serve as potential models in this system (many of which may also be new to science). Here I summarize some of the more salient results, while acknowledging that there is undoubtedly still more to learn about this fascinating system.

ESTABLISHING MIMICRY

In chapter II we addressed each of the requisites for establishing mimicry laid out by Roy and Widmer (1999), and our findings support the mimicry hypothesis. **1. Overlapping distribution and phenology:** We documented, with vouchered specimens, strongly overlapping distributions and phenology of *Dracula* with co-occurring mushrooms. **2. Require pollinators:** Hand pollinations of *D. felix* support the necessity of insect pollination for this species, with none of the unmanipulated autogamous

treatments setting fruit. **3. Individuals move between the organisms***:* In the experiment where we moved mushrooms into proximity of *Dracula* flowers and observed visitation, we documented 12.8% of visitors moving directly between orchids and mushrooms. In chapter IV we also discuss collections of pollinia-carrying flies directly from mushrooms, including individuals of *Hirtodrosophila* sp.7, *Zygothrica* sp.12, *Zygothrica* sp. 26, and *Zygothrica prensiseta*. The right panel in figure 2.1 shows an unidentified *Zygothrica* species carrying orchid pollinia while visiting a *Polyporus craterellus* (Polyporaceae RLC 717). **4. Similarity is important for fitness:** Again in chapter II, our experiments show that visitation rates of flies to *D. felix* flowers increased when plants were moved from isolation to close proximity with mushrooms. There was also no significant difference in the number of visits to flowers located next to flowers compared to those located next to mushrooms, suggesting that, functionally, the flies may not distinguish between the flowers and mushrooms. In one experiment we actually found more visits to the floral/fungal mixtures than to either type on its own (Figure 2.4b) suggesting some level of facilitation between the organisms. By showing that insect visitation and fruit set is higher in *Dracula* species than in co-occurring, closely-related taxa (*Masdevallia* species) that do not exhibit mushroom mimicry (Figure 2.2), we add further support to the case for mushroom mimicry being adaptive in this particular environment.

MECHANISMS OF ATTRACTION

In chapter III, we dissected the mechanisms of attraction in *Dracula* orchids and the artificial flowers were critical to decoupling sensory aspects of the unique *Dracula* phenotype. We found that the visual and olfactory aspects of the signaling phenotype are both important in the attraction of pollinating flies (Figure 3.3, Figure 3.4 & Figure 3.7).

Both visual and olfactory cues were sufficient to elicit an approach, however neither alone was adequate to provoke a landing (Figure 3.3a), but when both aspects were present there were more landings (Figure 3.3b). These data suggest a synergistic effect in the signaling phenotype.

The construction of chimeras from real and artificial parts allowed an even more nuanced look at visitor attraction and retention. Employing real labella and associated parts retained the majority of the native volatiles of the flower, while utilizing the real calyces enabled us to maintain the chemo-tactile sensory elements that would be very difficult to replicate. The most striking result here was that, not only did landings to the labellum drop off precipitously without a real labellum, but landings overall appeared to depend on the presence of the real labellum (Table 3.1). Flies did spend more time on the Real Calyx Chimera (which had an artificial labellum), but the activity was not directed, as the flies would visit the artificial labellum, but then return to the calyx, and perhaps back again. Visitation to the artificial flower was highly simplified with no visitors making any intra-floral movements. Because of the proximity of the column (fused reproductive structures) to the labellum, attraction to the labellum, and ultimately getting wedged up under the column, posterior to the labellum, is key to pollinia removal and deposition.

In the experiment on color, pattern and contrast, the contrasting treatments were more attractive than the solid colored treatments (Figure 3.7). These data suggest that the attractiveness of contrasting patterns may act at different scales. The ‘white’ artificial flower treatment, which displayed the strongest contrast against the background vegetation, received the least number of visits. The two treatments that displayed finer

scale contrast had visitation rates well above the solid treatments (Figure 3.7). It may be that smaller scale provides contrast at a level that the flies can perceive. An alternative explanation is that the small dark spots on a light background may look like other flies serving to attract additional individuals, acting as decoys (Johnson and Midgley 1997, pers. obs., and pers. com. D. Grimaldi). This (albeit untested) hypothesis suggests added sensory-mimetic complexity in an already extraordinary system.

In terms of the volatile components of *Dracula* flowers, two main features stand out. First, the volatile compound primarily responsible for the 'mushroomy' odor in *Dracula lafleurii* is chemically identical to that in the mushrooms themselves, the 8-carbon 'mushroom alcohol' 1-octen-3-ol. Second, the emission of this fungal volatile is restricted to the visibly mushroom-like labellum (Figure 3.8b).

It is clear that the integration of both visual and olfactory cues is critical to the success of *Dracula lafleurii* in attracting visitors. The same phenomenon has been found in other pollination systems, with each component on its own eliciting a subset of necessary behaviors, but both being required for complete pollinator attraction and visitation (Raguso and Willis 2002), so it is the cross-kingdom mimicry in both visual and chemical signals that makes this system remarkable.

We suspect that there are multiple layers to the mimicry. The fact that the part of the flower that looks like a mushroom is where the fungal volatiles are concentrated is intuitively satisfying, yet the large showy calyx also clearly plays a role in visitor attraction. This apparent disconnect in phenotype may explain some of the differences in host use by visitors that we document in chapter IV. Many of the visitors to *Dracula* flowers also are found on mushrooms, but a subset are not. These few taxa apparently

specialize on *Dracula* flowers, rather than being duped into visiting them by the resemblance to mushrooms. It is possible that the large showy calyx of many species of *Dracula* plays a role in attracting these non-mushroom associated flies.

HOST USE AND FLY BEHAVIOR

Fly visitation to *Dracula* does not simply reflect the overall diversity of the mycophilous or anthophilous groups of Drosophilidae; it is much more selective. Our data show considerable overlap in visitors to the orchid species with those to nearby mushrooms, although there also are unique visitors to each substrate as well.

Our malaise trap results further show that these flies are specific to mushrooms and mushroom-mimicking *Dracula* orchids, and not necessarily simply common in the habitat. In fly species where we could determine preference in host use, they ran the gamut, from mushroom or *Dracula* specialists exclusively, to mushroom or *Dracula* specialists that could be found on the other substrate at some frequency, to species like *Zygothrica prensiseta* that appear to be bona fide generalists.

The most notable result in terms of behavior by these flies was that they showed similar patterns of behavior at each substrate despite being generally more active on *D. lafleurii,* until we get to the rearing data. Importantly, 95% of all flies were reared from mushrooms, and no species that eclosed from of mushrooms also eclosed from *Dracula* flowers and vice versa (Table 4.1).

What are all these flies doing in the flowers? In terms of resource utilization, even when the flies are just standing within the calyx of the *Dracula* flowers, or on the underside of mushroom caps, they are sheltered from the rain. However, the apparent crowding at *Dracula* flowers suggests that shelter may be secondary compared with other

functions such as rendezvous sites. The role of mushrooms as rendezvous sites by flies in these groups is well documented and the wing-flicking behavior that we have documented has been suggested to play a role in courtship (Parsons 1977, Burla 1990). These observations suggest that the visitors in this study may indeed be utilizing both the flower and mushroom resources to obtain mates.

Our data also are interesting for the anthophilous Drosophilidae that were either rare or entirely absent from *Dracula*. These conspicuous absences suggest that *Dracula* is not simply exploiting known flower-breeding insects, despite the diverse flower-breeding species that have evolved in various groups of Drosophilidae (Frota-Pessoa 1952, Heed 1968, Carson and Hartt 1971, Montgomery 1975, Okada 1975, Carson and Okada 1980, Okada and Carson 1980, Brncic 1983), further suggesting a mushroom-mimicky strategy.

NATURE OF THE MIMETIC RELATIONSHIP & CONSERVATION IMPLICATIONS

Our data show that flies that breed in mushrooms are spending time on *Dracula* flowers, where they can move pollinia, but do not necessarily breed. These flies are still getting rewards from their visitation such as a mating site, shelter, and possibly grazing on superficial yeasts, so the deception may not lead to a total loss of fitness. Considering these factors, the relationship between *Dracula* orchids and mushrooms does not fit cleanly into a Batesian mimicry model, but falls somewhere along the continuum between Batesian (deception) and Müllerian (convergence) mimicry.

Like all mimicry systems, this one involves associations between multiple organisms (Roy and Widmer 1999), each of which is dependent on a particular set of habitat requirements. The cloud forests where this association is found (Luer 1993) provide the conditions necessary to support high biodiversity (Jorgensen and León-Yanez

1999), however they are under imminent threat of disappearing, even now covering less than a quarter of their original range (Myers et al. 2000). Locally at Reserva Los Cedros, logging, small-scale agriculture, international open-pit mining, and proposed hydroelectric projects all threaten the primary forest where these interactions are found.

As we continue to unravel the complexity of how this mushroom mimicry is achieved, it is the hope of all of us involved in this project that these unusual stories of natural history will inspire future efforts at conserving the unique habitats in which these organisms occur.

APPENDIX A

SUPPLEMENTAL FIGURES

Figure S3.1. Variation in *Dracula lafleurii*. As predicted by mimicry theory, we see tremendous variation in floral phenotype. Here we present some of the variation in size and coloration. Upper left: (flower BRL3.1) sepal area = 530 mm^2; 42.9% pigmented; labellum area = 30.6mm^2. Upper right (flower BRL2.2) sepal area = 1442.7 mm^2; 39.0% pigmented; labellum area =76.176mm^2. Lower left (flower RLC2.8) sepal area = 411.49 mm^2; 66.7% pigmented; labellum area = 46.71 mm^2. Lower right (flower RLC3.4) sepal area = 1303.1 mm^2; 81.9% pigmented; labellum area = 44.22 mm^2. Across 22 flowers sepal area ranged from 411-1623 mm^2; pigmentation ranged from 38-93%; and labella area ranged from16-97mm^2. Photos © B. A. Roy.

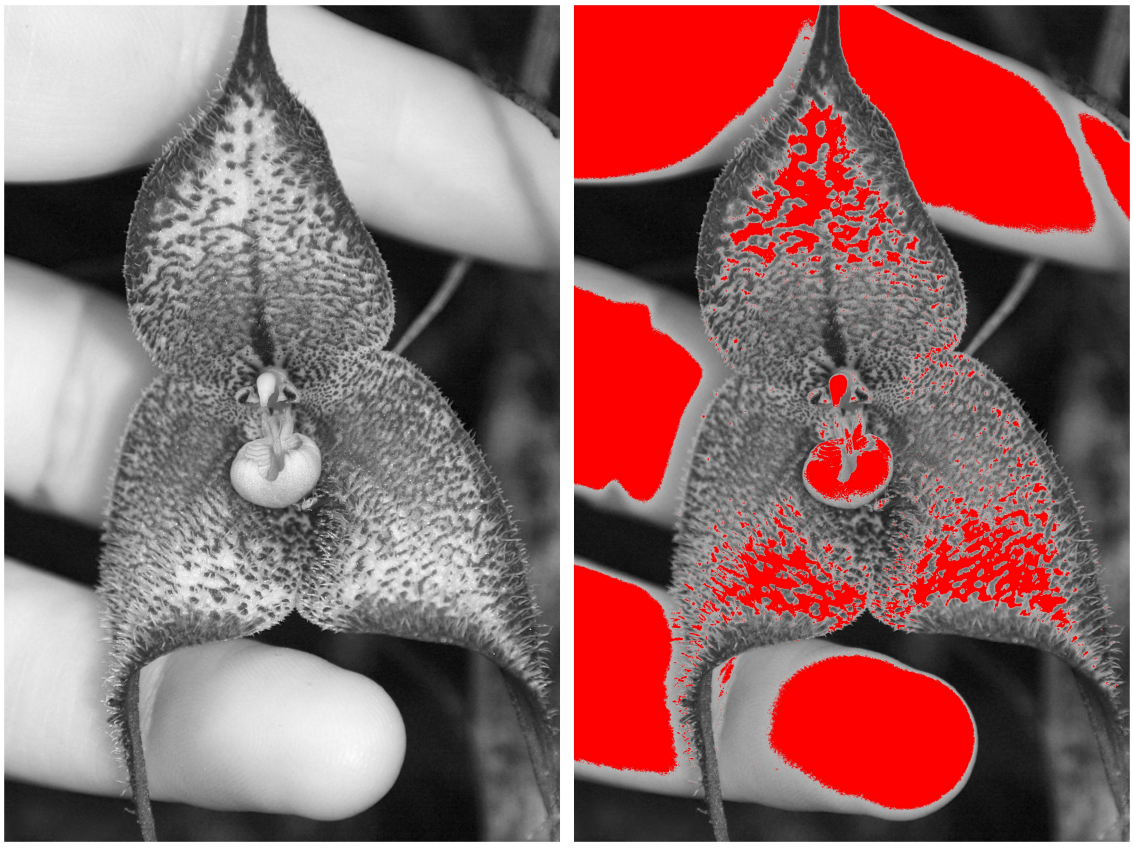

Figure S3.2. Measuring contrast in *Dracula lafleurii*. Flowers were photographed in the field and then analyzed in ImageJ (http://rsb.info.nih.gov). To determine the proportions of red, green and blue (RGB) in each flower image we used the color profiler of ImageJ after first outlining the flower using the drawing tool. To determine variation in contrast, the images were split into RGB colors, and the red was used for analysis. The light color threshold for the red image was set to 185, the measurement the scale was calibrated with actual measurements, and the overall area vs. area of light color of each flower was determined. This specimen was 18.1% light colored, or 81.9% pigmented. Pigmentation in sepals ranged from 38-93% (n=22 flowers). Photos © B. A. Roy.

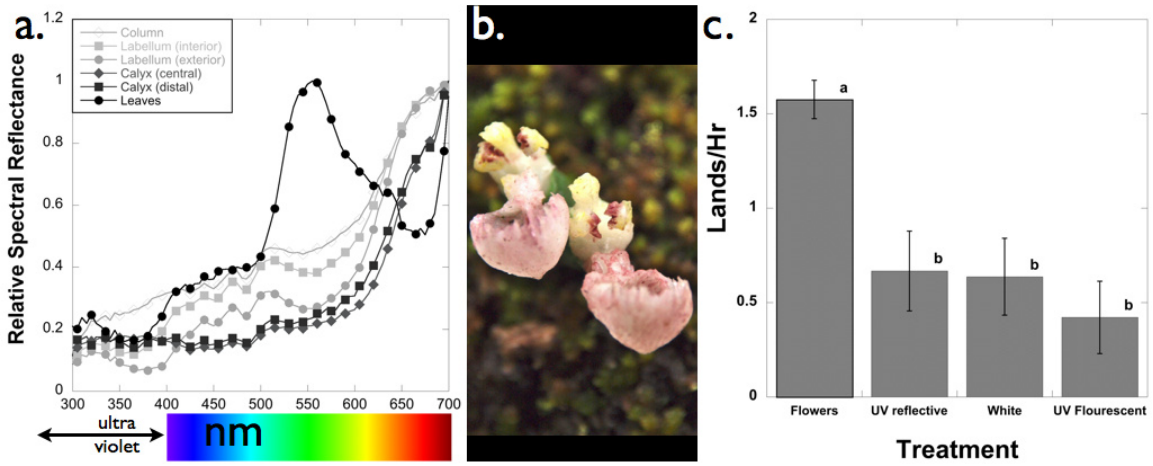

Figure S3.3. UV does not appear to play a strong role in attraction to *Dracula lafleurii*? a) To measure spectral reflectance (300-700 nm) we used a USB4000 miniature fiber optic spectrometer with a T300-RT-UV-VIS probe (Ocean Optics, Dunedin, FL, USA). We present the average of five readings per part of the flower [column, exterior surface of the labellum, surface interior of the labellum, central (ventral) calyx, distal (ventral) calyx and leaves]. Our measurements of UV reflectance do not indicate a strong UV signal in *D. lafleurii*. What little UV reflectance there is appears to come primarily from the column. Both surfaces of the labellum have slight peaks in the visible blue-green (400-550 nm). All portions of the flower had more pronounced peaks in the red part (600-700 nm) of the visual spectrum. **b)** Fabricated UV reflective, and UV florescent labella, used in the field trials. © B. A. Roy. **c)** To determine whether reflectance of UV was important for landings, three different kinds of artificial labella, UV-reflective, UV-fluorescent, and white, were inserted into color-matched artificial flowers and compared to a true flower (positive) control. N=eighteen 30 min time blocks (9 hours total). UV reflectance alone did not influence landings. While there was a treatment effect, flies visited the true flowers more often, and UV-reflective, UV-fluorescent, and white labella all received indistinguishable landing rates ($F_{3,127}$=14.15, P<0.0001). Diagram of visible spectrum in a) was modified and used by permission from V. Blacus under Creative Commons Attribution-Share Alike 3.0 Unported license.

Figure S4.1. Species of Dracula associated with depauperate insect collections. a) *Dracula morleyi*, photo © J. Poon. b) *D. cf. pubescens*, photo © J. Poon. c) *D. chiroptera,* photo © T. Policha. d) *D. sodiroi*, photo of flower © T. Policha, photo of the ligulate labellum © L. Baquero. Note the difference compared to the other species, in part the basis for inclusion in unique subgenus *Sodiroa* (Luer 1978).

Figure S4.2. Representatives of some of the mushrooms associated with insect collections. From left to right, top to bottom: *Mycena* (Mycenaceae) RLC 406 © M. Wherley, *Gerronema* (Marasmiaceae) RLC 805, *Marasmius* (Marasmiaceae) RLC 811, *Gerronema* (Marasmiaceae) RLC 824, *Collybia* (Tricholomataceae) RLC 829, *Filoboletus gracilis* (Mycenaceae) RLC 832, cf. *Pleurotus* (Pleurotaceae) RLC 930 © B. Roy, *Mycena* (Mycenaceae) RLC 1131 © B. Roy, *Mycena* (Mycenaceae) RLC TP9 © T. Policha. Photos © B. Dentinger unless otherwise noted.

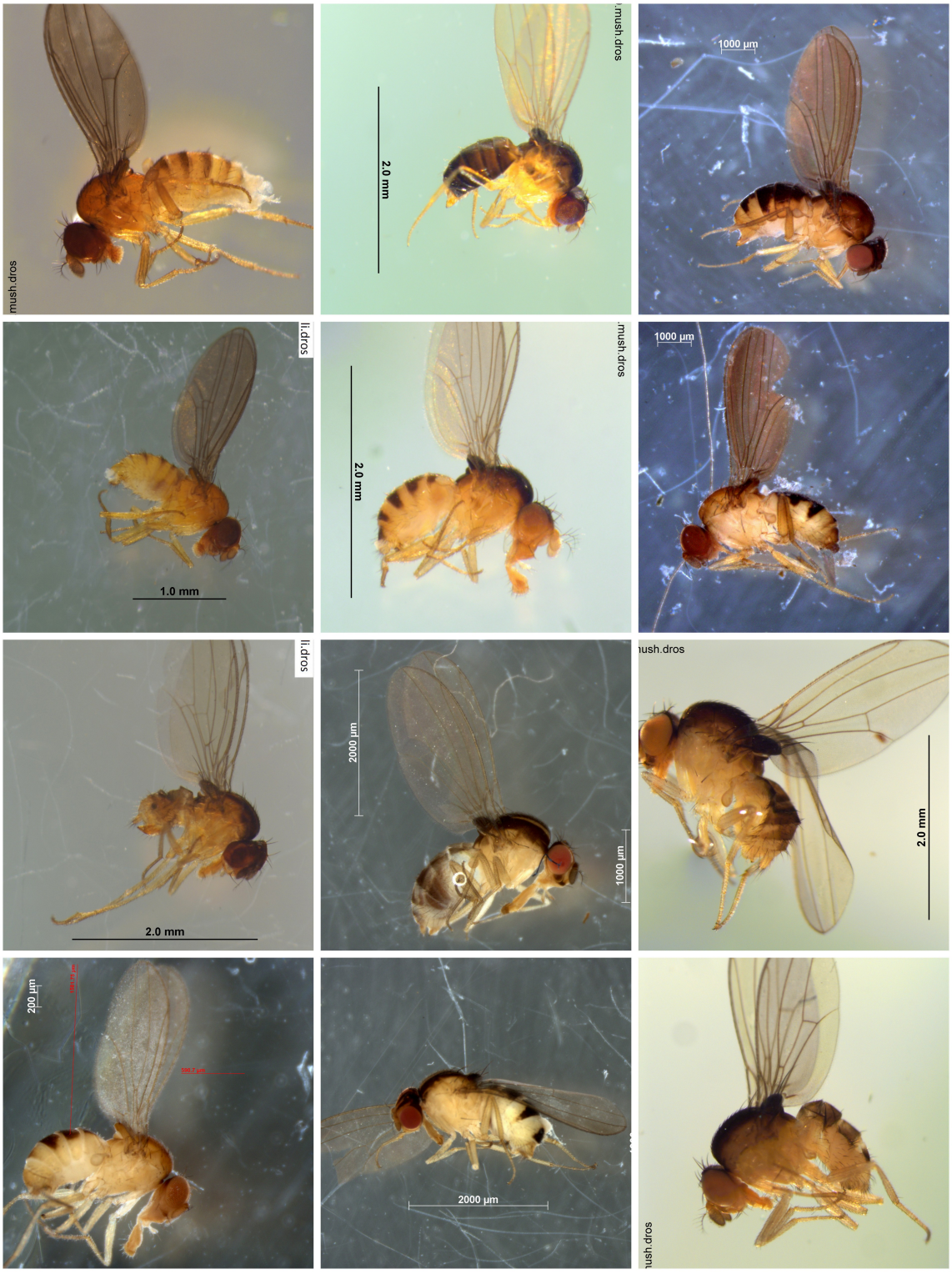

Figure S4.3a. Six common fly species. These are half of the species that make up the top twelve rows of Table 1 (listed alpha-numerically). The top two rows are *Hirtodrosohila sp. 6*, *Hirtodrosohila sp. 7*, and *Zygothrica sp. 3*. The bottom two rows are *Zygothrica sp. 6*, *Zygothrica sp. 8*, *Zygothrica sp. 9*. In all cases the female of the species is in the upper row and the male is directly below it in the lower row. Photos © A. Ludden, T. Policha, and B. Roy.

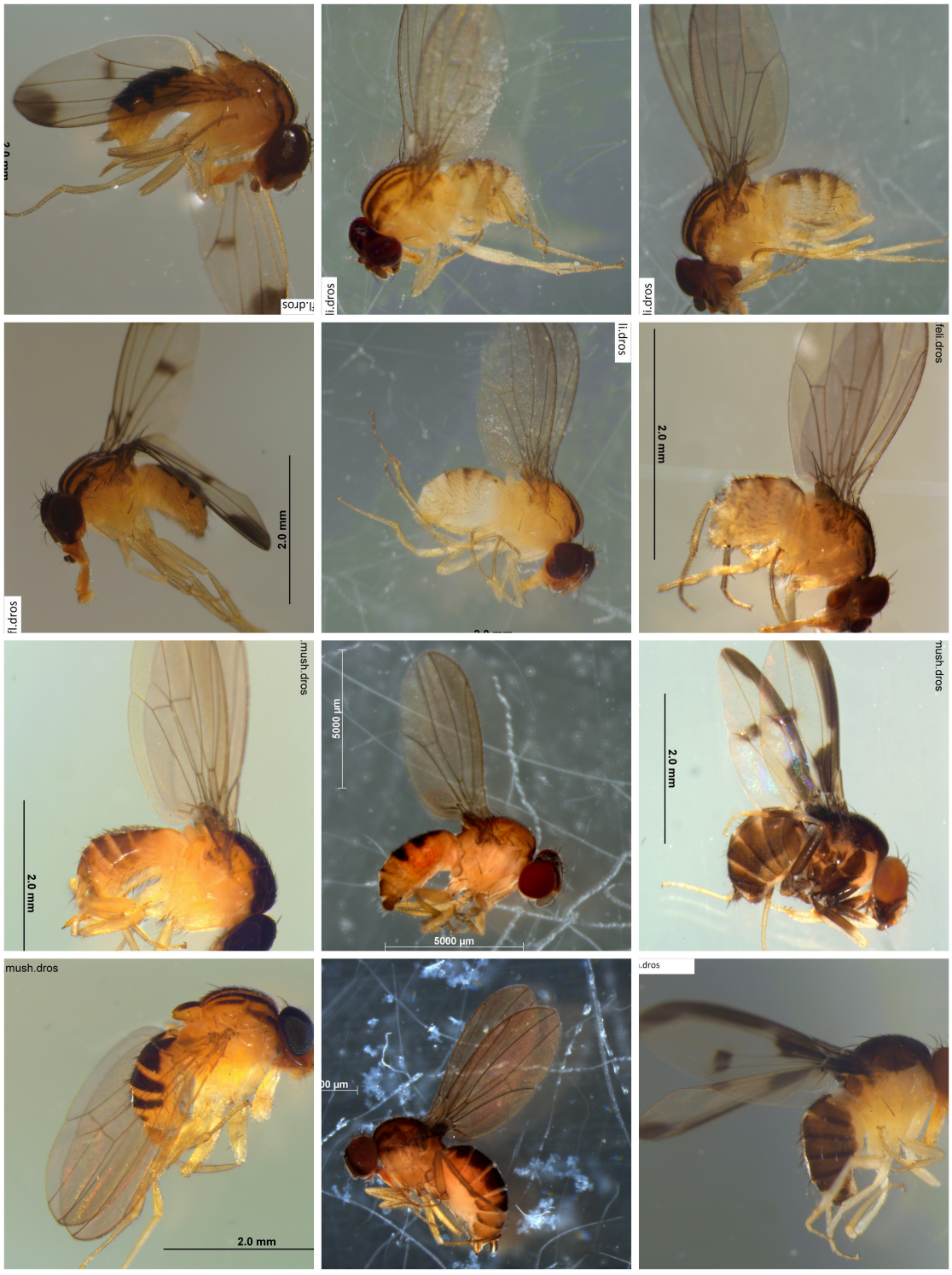

Figure S4.3b. Six common fly species. These are half of the species that make up the top twelve rows of Table 1 (listed alpha-numerically). The top two rows are *Zygothrica sp. 10*, *Zygothrica sp. 16*, and *Zygothrica sp. 20*. The bottom two rows are *Zygothrica sp. 29*, *Zygothrica sp. 41*, *Zygothrica preniseta*. In all cases the female of the species is in the upper row and the male is directly below it in the lower row. Photos © A. Ludden, T. Policha, and B. Roy.

APPENDIX B

PRESENCE/ ABSENCE DATA FOR THE EIGHT MOST COMMON CHEMICAL SPECIES

Sample Number	Group	Family	*Genus*	*Species*	Collection # (Fungi)	Part	Quantity	3 Octanone	3 Octanol	1 Octen 3 ol	Linalool	Methly Salicylate	Benzyl Alcohol	2 Phenylethanol	Eugenol
42	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Calyx	1		1	1		1	1		
60	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Calyx	1						1		
165	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Calyx	1					1			
45	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Column	1					1	1		
79	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Column	1					1	1		
137	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Column	1					1	1		
142	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Column	1					1			
168	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Column	1					1	1		
210	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Column	1					1	1		
213	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Column	1					1	1		
217	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Column	1					1	1		
225	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Column	1					1			
44	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Labellum	1			1			1	1	
78	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Labellum	1		1	1					

136	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Labellum	1			1					
141	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Labellum	1						1		
209	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Labellum	1		1	1			1		
212	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Labellum	1		1	1					
216	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Labellum	1						1		
224	Dracula	Orchidaceae	*Dracula*	*cf.pubescens*		Labellum	1		1	1			1		
28	Dracula	Orchidaceae	*Dracula*	*felix*		Whole	7			1			1	1	
184	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1					1			
200	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1					1			
324	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1					1			
328	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1					1			
332	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1						1		
344	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1					1	1		
348	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1					1			
352	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1					1			
360	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1					1			
364	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1						1		
368	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1						1		
378	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1					1			
386	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1						1		
390	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Calyx	1			1		1			
70	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1			
75	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1	1		

175	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1	1		
187	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1			
191	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1			1			1		
199	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1	1		
203	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1	1		
250	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1	1		
327	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1			
331	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1	1		
347	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1			
351	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1	1		
355	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1			
359	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1			
367	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1			
371	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1			
385	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1	1		
389	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1	1		
393	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Column	1					1			
69	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1					
74	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
174	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
186	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1	1	
198	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1	1	
202	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1						1		

249	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1	1	
326	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1						1		
330	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
334	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
346	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
350	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			**1**			1		
354	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1						1		
358	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1						1		
362	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
366	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
370	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1						1		
374	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1						1		
380	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
388	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
392	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Labellum	1			1			1		
173	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Petals	2						1	1	
349	Dracula	Orchidaceae	*Dracula*	*lafleurii*		Petals	2						1		
123	Dracula	Orchidaceae	*Dracula*	*morleyii*		Calyx	1					1		1	
127	Dracula	Orchidaceae	*Dracula*	*morleyii*		Calyx	1							1	
218	Dracula	Orchidaceae	*Dracula*	*morleyii*		Calyx	1							1	
232	Dracula	Orchidaceae	*Dracula*	*morleyii*		Calyx	1					1		1	
239	Dracula	Orchidaceae	*Dracula*	*morleyii*		Calyx	1					1		1	
50	Dracula	Orchidaceae	*Dracula*	*morleyii*		Column	1					1			

126	Dracula	Orchidaceae	*Dracula*	*morleyii*		Column	1					1			
130	Dracula	Orchidaceae	*Dracula*	*morleyii*		Column	1					1	1		
221	Dracula	Orchidaceae	*Dracula*	*morleyii*		Column	1					1			
235	Dracula	Orchidaceae	*Dracula*	*morleyii*		Column	1					1	1		
242	Dracula	Orchidaceae	*Dracula*	*morleyii*		Column	1					1			
49	Dracula	Orchidaceae	*Dracula*	*morleyii*		Labellum	1			1			1	1	
125	Dracula	Orchidaceae	*Dracula*	*morleyii*		Labellum	1			1			1	1	
129	Dracula	Orchidaceae	*Dracula*	*morleyii*		Labellum	1			1			1	1	
220	Dracula	Orchidaceae	*Dracula*	*morleyii*		Labellum	1			1			1	1	
234	Dracula	Orchidaceae	*Dracula*	*morleyii*		Labellum	1		1	1			1	1	
241	Dracula	Orchidaceae	*Dracula*	*morleyii*		Labellum	1			1			1	1	
124	Dracula	Orchidaceae	*Dracula*	*morleyii*		Petals	2						1	1	
128	Dracula	Orchidaceae	*Dracula*	*morleyii*		Petals	2						1	1	
219	Dracula	Orchidaceae	*Dracula*	*morleyii*		Petals	2						1		
233	Dracula	Orchidaceae	*Dracula*	*morleyii*		Petals	2						1		
121	Dracula	Orchidaceae	*Dracula*	*morleyii*		Whole	1	1		1		1	1	1	
122	Dracula	Orchidaceae	*Dracula*	*morleyii*		Whole	1			1			1	1	
133	Dracula	Orchidaceae	*Dracula*	*morleyii*		Whole	1			1			1	1	
159	Dracula	Orchidaceae	*Dracula*	*morleyii*		Whole	1	1		1		1	1	1	
160	Dracula	Orchidaceae	*Dracula*	*morleyii*		Whole	1	1		1		1	1	1	
161	Dracula	Orchidaceae	*Dracula*	*morleyii*		Whole	1			1		1	1	1	
151	Fungi	Agaricaceae	*Lepiota*		713	Whole	1			1					
90	Fungi	Cortinariaceae	*Gallerinoid*		637	Whole	1	1		1				1	

24	Fungi	Cortinariaceae	*Gymnopilus*		408	Whole	1	1		1					
29	Fungi	Inocybaceae	*Crepidotus*		404	Whole	2							1	
30	Fungi	Inocybaceae	*Crepidotus*		404	Whole	1							1	
31	Fungi	Inocybaceae	*Crepidotus*		404	Whole	1							1	
143	Fungi	Mycenaceae	*Filoboletus*			Pileus	Part			1					
109	Fungi	Mycenaceae	*Filoboletus*		661	Whole	1							1	
118	Fungi	Pleurotaceae	*Pleurotus*		668	Whole	1			1					
65	Fungi	Polyporaceae	*conk*		413	Sporocarp	Part	1		1					
98	Fungi	Polyporaceae	*ganodermoid*		645	Sporocarp	Part			1					
52	Fungi	Polyporaceae	*Polystictus*		410	Pileus	Part	1	1	1				1	
41	Fungi	Polyporaceae	*Polystictus*		409	Whole	1	1		1					
147	Fungi	Polyporaceae	*Polystictus*		698	Whole	1	1	1	1				1	
93	Fungi	Tricholomataceae	*Hypsizygus*		640	Whole	1			1					
23	Fungi	Tricholomataceae			407	Whole	1			1					
110	Fungi	unknown				Whole	2	1		1					
106	Fungi	unknown 1	*parasitized agaric*		653	Whole	1	1		1				1	
107	Fungi	unknown 1	*parasitized agaric*		653	Whole	2							1	
229	Fungi	unknown 4			721	Whole	1	1		1				1	
252	Fungi	unknown 5				Whole	1							1	
238	Fungi	unknown 6			722	Whole	1			1					
180	Orchids	Orchidaceae	*Brachionidium*	*ingramii*		Whole	1								1
85	Orchids	Orchidaceae	*Masdevallia*	*nidifica*		Whole	2						1		
86	Orchids	Orchidaceae	*Masdevallia*	*nidifica*		Whole	2						1		

87	Orchids	Orchidaceae	*Masdevallia*	*nidifica*		Whole	2						1		
88	Orchids	Orchidaceae	*Masdevallia*	*nidifica*		Whole	2						1		
104	Orchids	Orchidaceae	*Masdevallia*	*nidifica*		Whole	2						1		
114	Orchids	Orchidaceae	*Masdevallia*	*ximenae*		Whole	1				1			1	1
115	Orchids	Orchidaceae	*Masdevallia*	*ximenae*		Whole	1				1	1		1	1
177	Orchids	Orchidaceae	*Masdevallia*	*ximenae*		Whole	1				1	1		1	
178	Orchids	Orchidaceae	*Masdevallia*	*ximenae*		Whole	1				1			1	
179	Orchids	Orchidaceae	*Masdevallia*	*ximenae*		Whole	1				1	1		1	1
192	Orchids	Orchidaceae	*Masdevallia*	*ximenae*		Whole	1				1			1	1
193	Orchids	Orchidaceae	*Masdevallia*	*ximenae*		Whole	1				1	1		1	1
164	Orchids	Orchidaceae	*Poroglossum*	*hoeijeri*		Whole	1						1		
204	Orchids	Orchidaceae	*Scaphosepalum*			Whole	1						1		
246	Orchids	Orchidaceae	*Scaphosepalum*			Whole	1						1		
138	Orchids	Orchidaceae	*Trisetella*	*dalstroemii*		Whole	1					1			

Shaded columns are typical mushroom volatiles, unshaded columns are typical floral volatiles. Only the data for *Dracula lafleurii* were included in the analysis presented in the text, but the other *Dracula* species are included here for comparison. For the comparisons of *D. lafluerii* to mushrooms and the other orchids the dissected flower parts were combined so that each replicate represented the volatiles from a single flower. Notably, *D. cf. pubescens* and *D. morleyi* produce the mushroom volatiles 3-octanone and 3-octanol, which were not detected in *D. lafluerii*. Samples were compared against known standards for these eight compounds.

APPENDIX C

SUMMARY TABLE OF ALL IDENTIFIED DROSOPHILIDS

Flies		Substrates			Collections									Reared								All
Genus	*species*	*Dracula* spp.	Mushrooms	SHARED	Mushrooms (30)	*D. chiroptera (1)*	*D. felix (41)*	*D. lafleurii (19)*	*D. morleyi (6)*	*D. pubescens (6)*	*D. sodoroi (1)*	Control flowers (1)	Collection Totals	Mushrooms (46)	*D. felix (14)*	*D. lafleurii (8)*	*D. morleyi (10)*	*D. pubescens (3)*	Control flowers (10)	Reared Totals	Malaise Trap	ALL TOTALS
Cladochaeta	*A*	1						1					**1**		2	1				**3**	2	**6**
Diathoneura	*spp.*	1											**0**		2		2	4		**8**		**8**
Drosophila	*1*		1		4								**4**							**0**		**4**
Drosophila	*2*		1		1								**1**							**0**		**1**
Drosophila	*3*												**0**						2	**2**		**2**
Drosophila	*4*	1											**0**			1				**1**		**1**
Drosophila	*5*												**0**							**0**	1	**1**
Drosophila	*A*		1		1								**1**							**0**		**1**
Drosophila	*B*											2	**2**							**0**		**2**
Drosophila	*C*											1	**1**							**0**		**1**
Drosophila	*New*		1		4								**4**							**0**		**4**
Drosophilidae	*novo genus*		1		1								**1**							**0**		**1**
Hirtodrosophila	*1*	1	1	**1**	2		1						**3**							**0**		**3**
Hirtodrosophila	*2*	1	1	**1**	3		2						**5**							**0**		**5**

Hirtodrosophila	*3*		1		3							**3**			**0**	**3**
Hirtodrosophila	*4*		1		1							**1**			**0**	**1**
Hirtodrosophila	*5*		1		1							**1**			**0**	**1**
Hirtodrosophila	*6*	1	1	**1**	9	1	4					**14**	4		**4**	**18**
Hirtodrosophila	*7*	1	1	**1**	25		1					**26**	27		**27**	**53**
Hirtodrosophila	*8*	1	1	**1**	3		1					**4**	6		**6**	**10**
Hirtodrosophila	*9*	1	1	**1**	3		2					**5**	6		**6**	**11**
Hirtodrosophila	*10*		1		3							**3**			**0**	**3**
Laccodrosophila	*1*	1								2		**2**			**0**	**2**
Laccodrosophila	*3*	1					2		8			**10**		6	**6**	**16**
Zapriothrica	*1*	1									1	**1**			**0**	**1**
Zygothrica	*aliucapa*	1						1				**1**			**0**	**1**
Zygothrica	*caputrichia*		1		2							**2**			**0**	**2**
Zygothrica	*prensiseta*	1	1	**1**	4		3	2				**9**			**0**	**9**
Zygothrica	*1*	1					2					**2**			**0**	**2**
Zygothrica	*2*	1	1	**1**	2		2	1				**5**	15		**15**	**20**
Zygothrica	*3*	1	1	**1**	2		22					**24**			**0**	**24**
Zygothrica	*4*	1						5				**5**			**0**	**5**
Zygothrica	*5*	1	1	**1**	1		1	3				**5**			**0**	**5**
Zygothrica	*6*	1					17	16				**33**			**0**	**33**
Zygothrica	*8*	1	1	**1**	11			3				**14**			**0**	**14**
Zygothrica	*9*	1	1	**1**	17		1	1				**19**	5		**5**	**24**

Zygothrica	*10*		1					29			**29**			**0**	**29**
Zygothrica	*12*			1		8					**8**	2		**2**	**10**
Zygothrica	*13*			1		2					**2**			**0**	**2**
Zygothrica	*14*			1		1					**1**			**0**	**1**
Zygothrica	*15*			1		1					**1**			**0**	**1**
Zygothrica	*16*		1				29	1			**30**			**0**	**30**
Zygothrica	*17*			1		1					**1**			**0**	**1**
Zygothrica	*18*		1				2	1			**3**			**0**	**3**
Zygothrica	*19*		1				1				**1**			**0**	**1**
Zygothrica	*20*		1	1	**1**	2	14	1			**17**	9		**9**	**26**
Zygothrica	*21*		1	1	**1**	1	2	1			**4**			**0**	**4**
Zygothrica	*22*		1				1				**1**			**0**	**1**
Zygothrica	*23*		1	1	**1**	1	1				**2**			**0**	**2**
Zygothrica	*24*		1				2				**2**			**0**	**2**
Zygothrica	*25*		1					3			**3**			**0**	**3**
Zygothrica	*26*		1	1	**1**	6		1		3	**10**			**0**	**10**
Zygothrica	*29*		1	1	**1**	15	5			1	**21**			**0**	**21**
Zygothrica	*30*		1	1	**1**	3				1	**4**			**0**	**4**
Zygothrica	*31*		1	1	**1**	1	1				**2**			**0**	**2**
Zygothrica	*32*			1		3					**3**			**0**	**3**
Zygothrica	*33*		1	1	**1**	1		1			**2**			**0**	**2**
Zygothrica	*34*		1					1			**1**			**0**	**1**

Zygothrica	*35*		1						1				**1**									**0**		**1**
Zygothrica	*36*		1	1	**1**			1					**1**	2								**2**		**3**
Zygothrica	*38*		1	1	**1**	1				1			**2**									**0**		**2**
Zygothrica	*41*		1	1	**1**	32		3					**35**	98								**98**		**133**
Zygothrica	*42*			1		1							**1**									**0**		**1**
Zygothrica	*43*			1		1							**1**									**0**		**1**
Zygothrica	*45*			1		1							**1**									**0**	1	**2**
Zygothrica	*46*			1		5							**5**	29								**29**		**34**
Zygothrica	*47*			1		1							**1**	2								**2**		**3**
Zygothrica	*48*			1		9							**9**	4								**4**		**13**
Zygothrica	*49*		1	1	**1**	2		1					**3**									**0**		**3**
Zygothrica	*50*			1									**0**	3								**3**		**3**
Zygothrica	*51*			1		1							**1**									**0**		**1**
Zygothrica	*52*		1						1				**1**									**0**		**1**
Zygothrica	*53*		1	1	**1**			1					**1**	1								**1**		**2**
Zygothrica	*54*		1				1						**1**									**0**		**1**
Zygothrica	*55*		1						1				**1**									**0**		**1**
Zygothrica	*56*		1	1	**1**	2		1					**3**									**0**		**3**
Zygothrica	*57*		1	1	**1**	1			3				**4**									**0**		**4**
		TOTALS	**49**	**51**	**27**	**206**	**2**	**126**	**78**	**9**	**7**	**1**	**3**	**432**	**213**	**4**	**2**	**8**	**4**	**2**	**233**	**4**	**669**

Summary table of all identified drosophilids from wild collections, rearing studies and the passive malaise trap. Species are sorted alpha-numerically. The 3 columns labeled “Substrates” represent presence absence data, all other cells are counts of individuals (n=669).

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