NOCTURNAL POLLINATION BY FUNGUS GNATS
OF THE COLOMBIAN ENDEMIC SPECIES, *PLEUROTHALLIS MARTHAE* (ORCHIDACEAE: PLEUROTHALLIDINAE)

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Abstract. Contemporary patterns of plant biodiversity result from the ecological and evolutionary processes generated by species interactions. Understanding these interactions is key for effective biodiversity conservation at the species and the ecosystem level. Orchid species often have highly specialised pollinator interactions, and the preservation of these is critical for in situ orchid conservation. The majority of orchid species occur in tropical regions, and information regarding their interactions is limited. We present data on pollinator identities, pollination mechanisms and flowering phenology of the Colombian endemic orchid, *Pleurothallis marthae*. We evaluated the mechanisms of attraction, the presence of osmophores, and the reproductive system of the species. *Pleurothallis marthae* is self-compatible with nocturnal anthesis pollinated by *Mycetophila* sp. (Mycetophilidae), probably attracted by a string fungus like smell liberated by the flower and *Bradysia* sp. (Sciaridae) that feed on nectar in the labellum. Osmophores and nectaries were detected in the epidermis of the sepals and petals. We present new evidence that the genus *Pleurothallis* is adapted to Diptera pollination. Our study indicates that the pollination mechanism of *P. marthae* is based on the nocturnal attraction of two species of fungus gnats, probably combining food attraction and brood place deception.

Resumen. Los patrones contemporáneos de biodiversidad vegetal son el resultado de procesos ecológicos y evolutivos generados por la interacción entre especies. El entendimiento de estas interacciones es clave para la conservación de la biodiversidad a nivel de especies y ecosistemas. A menudo las orquídeas tienen interacciones de polinización altamente especializadas, y su preservación es crucial para la conservación de orquídeas *in situ*. La mayoría de las especies de orquídeas ocurren en regiones tropicales, y la información sobre sus polinizadores es limitada. Aquí presentamos datos sobre la identidad de polinizadores, mecanismos de polinización y fenología floral de la orquídea *Pleurothallis marthae*, endémica de Colombia. Evaluamos los mecanismos de atracción, la presencia de osmóforos, y su sistema reproductivo. Encontramos que se trata de una especie auto-compatible con antesis nocturna polinizada por *Mycetophila* sp. (Mycetophilidae), especie atraída probablemente por el olor a hongo que libera la orquídea y *Bradysia* sp. (Sciaridae), la cual se alimenta de gotas de néctar en el labelo. Los osmóforos y nectarios fueron detectados en la epidermis de los sépalos y pétalos. El mecanismo de polinización de *P. marthae* se basa en la atracción nocturna de moscas especializadas en hongos, y combina atracción alimenticia y mimetismo de sitios de apareamiento.

Key words: *Bradysia*, orchid pollination, fungus gnats, pollinators, *Mycetophila*, olfactory mimicry

Introduction. As we are facing the sixth global biodiversity extinction (Canadell & Noble 2001), the ultimate goal of plant conservation biology is to preserve an adequate environment in which species can persist (Swarts & Dixon 2009), with continued evolutionary change. The effective conservation of individual species requires a deep taxonomic understanding (Flanagan *et al.* 2006), especially among those groups highly diversified such as Pleurothallidinae. It also demands the maintenance of species interactions in natural environments. One of the critical interactions for many plants is pollination by animals. It is also

one of the interactions that have provided substantial evidence of evolutionary processes in the wild; revealing amazing adaptations associated with pollen transport (Darwin 1885). Nevertheless, pollination had been nominated as an endangered interaction due to losses of plant species and their pollinators as well as their natural habitats (Kearns *et al.* 1993). A flowering plant family that combines both, threat conservation in some species and interesting pollination interactions is the Orchidaceae.

Orchidaceae is the largest family of flowering plants with about 800 genera and more than 28,000 species (Govaerts *et al.* 2012); many species are endangered, and listed in biodiversity red lists; for example, in Colombia there is a partial list of 207 endangered orchids (Calderón 2007). There are several critical aspects of orchid biology that contribute to those threats. Orchid conservation requires the consideration of two bottlenecks in the orchid's life cycle: seed germination and pollination (Tremblay & Otero 2009). Orchids depend on specific fungi for seed germination (Otero *et al.* 2002; 2004; 2007) that may have had complex co-cladogenic processes (Otero *et al.* 2011); however, there is still much to learn about many orchid mycorrhizal interactions, especially in the tropics (Otero & Bayman 2007). In the Andes, the highest threat to orchid conservation is habitat loss; nevertheless, pollination is also crucial.

Orchids are renowned for the complex and intriguing pollination mechanisms adapted to pollination by animals (Darwin 1885, van der Pijl & Dodson 1966, Singer 2002, Borba & Braga 2003), especially insects (Dressler 1981). It is now well known that bees and wasps are responsible for about 60% of the pollination of the family (Williams 1982, Ackerman 1983; Whitten *et al.* 1993; Camargo *et al.* 2006), flies pollinate 15-25% of species (Borba & Semir 2001), and Lepidoptera, Coleoptera and birds pollinate about 15-25% remaining (van der Pijl & Dodson 1966; Singer *et al.* 2007; Cuartas-Domínguez & Medel 2010). Orchids may provide a variety of rewards to their pollinators, such as oils, floral fragrances and occasionally pollen-like substances (Neiland & Wilcock 1998; Pansarin 2008); however, nearly a third of orchid species provide no floral resources, and depend on various forms of deception for pollination (Ackerman 1986; Nilsson 1992; Cozzolino & Widmer 2005). One of the

most common means of deception, sexual mimicry, is reported in about 20 genera of Orchidaceae with various pollinator types such as solitary and social bees, wasps, beetles and flies (Dafni 1984; Sasaki *et al.* 1991). This mechanism includes visual and/or olfactory features attracting insects. The best known cases of reproductive deception involve pseudo-copulation, a phenomenon unique to orchids (Nilsson 1992). Where, flowers mimic female structures and chemical pheromones of certain insects (Singer *et al.* 2004; Blanco & Barboza 2005), and are pollinated by male insects seeking a mate (Dressler 1981; Williams & Whitten 1983; Singer 2002). The emission of fragrances that resemble the insect sexual pheromones for reproduction is an important factor associated with the mimicry, including mating or oviposition sites (Albores-Ortiz & Sosa 2006; Barbosa *et al.* 2009; Barriault *et al.* 2010; Endara *et al.* 2010 Peakall *et al.* 2010).

Some studies suggest that the subtribe Pleurothallidinae (Orchidaceae: Epidendreae) is mainly adapted to pollination by Diptera species (van der Pijl & Dodson 1966; Chase 1985; Duque 1993; Borba & Semir 2001; Blanco & Barboza 2005; Pupulin *et al.* 2012), with several reports of deception by sexual mimicry (Christensen 1994; Blanco & Barboza 2005). Borba & Semir (2001) studied four Brazilian species and reported different fragrances and pollinator activities. *Acianthera johannensis* (Barb. Rodr.) Pridgeon & M.W. Chase (as *Pleurothallis johannensis* Barb. Rodr.) and *Acianthera fabiobarrosii* (Borba & Semir) F. Barros & F. Pinheiro (as *P. fabiobarrosii* Borba & Semir) are pollinated by females of the genera *Tricimba* (Chloropidae) and have a diurnal smell of fish and no nectar production, while *Acianthera teres* (Lindl.) Borba (as *P. teres* Lindl.) and *Acianthera ochreata* (Lindl.) Pridgeon & M.W. Chase (as *P. ochreata* Lindl.) have a diurnal smell of rancid cheese and produce nectar like liquid at the base of the labellum. The latter are pollinated by *Megaselia* spp. (Phoridae) (Borba & Semir 2001), *Acianthera adamantinensis* (Brade) F. Barros (as *Pleurothallis adamantinensis* Brade) has a dog feces odor and is pollinated by flies *Hippelate* ssp. (Chloropidae) (Borba & Semir 2001). Those five species had high genetic variability showing a high percentage of polymorphic loci ranged from 50 to 83%, with a mean number of alleles per locus between 2.1 and 3.8, and a mean

heterozygosity (He) ranged from 0.25 to 0.43 (Borba *et al.* 2001). *Acianthera johannensis*, *A. fabiobarrosii*, and *A. adamantinensis* showed a low level of genetic structuring while *A. teres* and *A. ochreata* showed high genetic structuring (Borba *et al.* 2001). However, the actual evidence has not been applied for conservation purposes, but in recent years the genus had been subdivided in smaller groups.

Pleurothallis marthae (Luer & Escobar) Luer is an interesting research model because it has relatively large flowers of 2 cm that facilitate observation and is a frequent species in Yotoco Nature Reserve, Colombia. Little information is reported in the literature on this species. It is listed in the CITES Appendix II (W3Tropicos, accessed on Dec 2012) and is endemic to Colombia. Recently it was recorded from Yotoco Nature Reserve (Escobar 2001; Pérez-Escobar *et al.* 2009). The species grows as both an epiphyte near the ground (no more than one meter high) and a terrestrial plant, at 1400-1800 m elevation in mountain forest. The objectives of this study are to describe the pollination system and mechanisms of pollinator attraction of *P. marthae*.

Material and Methods

Study species. — *Pleurothallis marthae* is a terrestrial species or rarely epiphytic, without pseudobulbs, leaves broad, deeply cordate (Luer & Escobar 1996), it has a little bundle of pink flowers on the base of the leaf. The flowers are relatively large for the genus. This species was described as *P. marthae* by Luer & Escobar from a collection by E. Valencia from Garrapatas (Valle del Cauca, Colombia) (Luer & Escobar 1996) and then transferred to *Acronia* Luer (2005). As *Acronia* is not broadly accepted we use *Pleurothallis*. *P. marthae* has flowers with two different coloration patterns and morphology (morphs). The first (yellow-morph) has yellowish dorsal sepal the broadly ovate, obtuse, concave, with translucent horizontal lines and dots, the lateral sepals completely connate (synsepal) is lilac, with glandular trichomes, petals rose, concave, with glandular trichomes too, lip light rose, thick, convex, transversely obovate. The second (pink-morph) has a pink dorsal sepal with translucent horizontal lines and dots, the synsepal is magenta with glandular trichomes, the petals are larger than the yellow-morph (Fig. 1) and the lip is similar to yellow-morph. In both morphs

the column is short and cream-colored with an apical anther and a bilobed stigma (Fig. 1).

Study site. — We did this study at the Yotoco Forest Reserve (YFR), located on the eastern slope of the western Cordillera de Los Andes in Colombia, in the municipality of Yotoco. The site was situated in remnants of the subtropical wet-dry transition forest of Valle del Cauca (3°50'N, 76°20'W) at an elevation of 1400-1600 m. Average annual temperature is 20° C; average annual precipitation is 1500 mm; and relative humidity averages 85% (Escobar 2001).

Two populations were surveyed, accounting 250 individuals in total; each individual was marked with an aluminum tag with a previously assigned code. Individuals were considered genets if all shoots were attached to a single rhizome and were physically separate from other plants. Individuals were characterized by size class according to the length of the longest shoot. The classes are the following: SIZE I (6-20 cm), SIZE II (21-40 cm), SIZE III (41-60 cm), SIZE IV (> 61 cm).

Reproductive events. — The numbers of open flowers (OF), pollinaria removed (PR), closed stigmas (CS) and swollen ovaries (SO) were recorded weekly as indirect evidence of reproductive effort and pollination events. As the data were not normally distributed, the differences between size classes for each reproductive event were analyzed using a Kruskal-Wallis test. The correlation between pollinator activity and flowering anthesis was evaluated with a linear regression. All data were analyzed with Statgraphics ® Plus Version 5.1®.

Flower visits. — Observations were initially made between 0600 and 1800 h to identify periods of activity of flower visitors and pollinators. Very low activity was observed during the day, so the observation period were extended until 2000 h. Visitors were observed using red light located two meters from the plants to broaden the spectrum of light to avoid pollinator disturbance they were highly sensitive to direct illumination. With these additional hours we discovered visitation was nocturnal, so all further nocturnal observation periods were done between 1800 - 0600 h, for a total of 97 hours of observation (not including the diurnal times). For 30 individuals, the time of arrival of visitors was recorded, duration of visit, behavior within the flower, and number of visitors, and additionally video and

FIGURE 1. Flower of *Pleurothallis marthae*: a) lateral view, b) frontal view, c) two color morphs, d) Habit of *Pleurothallis marthae*.

photographic records. Flower visitors and pollinators were differentiated by behavior. Floral visitors were collected in plastic vials, transported to the zoology laboratory at Universidad of Caldas where high resolution macro photos were taken. As Colombian law prohibits exportation of wild biological material, Dr Sarah Siqueira de Oliveira, at the University of São Paulo identified the specimens from the photographs.

Osmophore detection.. — From each of the populations, we collected fresh flowers from 10 individuals and stained in a bath of neutral red 1:1000 for 20 minutes (Stern *et al.* 1986) to detect scent-secreting glands (osmophores) or nectaries as neutral red indicate sites that is metabolically active (Kearns & Inouye 1993).

Reproductive system. — We bagged 40 flower buds of 20 plants with net mesh to exclude insect visitors. Once the flowers were in anthesis and receptive, as indicated by a wet and sticky stigmatic surface, we performed

the following hand-pollination treatments: autogamy (removal of pollinaria from a flower and pollen transfer to the same flower); geitonogamy (removal of pollinaria from one flower and pollen transfer to another flower of the same plant); xenogamy (removal of pollinaria from a flower and pollen transfer to a flower of another plant); and natural pollination. All treatments with the exception of the natural pollination treatment were bagged again to prevent insect visitors impacting the results. Pollinations were monitored until fruits matured or aborted. We analyzed differences among treatments with a non-parametric Kruskal-Wallis test.

Results. Flower buds develop in 10-15 days, and flowers remain open for 10-12 days, provided that pollination did not occur. The flowers produce fungus-like odor and lightly sweet nectar at night time. Anthesis of *P. marthae* began at 18:30 when flowers began to release a fungus-like odor (as detected by the human
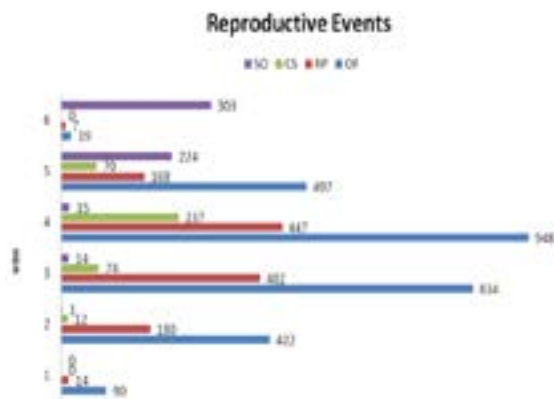
FIGURE 2. Linear regression of the number of flowers per reproductive season vs. number of fruits.

FIGURE 3. *Bradysia* sp. with *Pleurothallis marthae* pollinaria adhered to the dorsal side of the thorax.

nose) and nectar secreted by the lip. As time passed the smell gradually increased in intensity, and peaked at 23:30. There after the intensity decreased until approximately 05:00, when it became imperceptible. The fluid secreted from the lip remained in the flower from 19:00 until 09:00.

Reproductive events. — The species flowered from early May through mid June (six weeks), with a peak at the fourth week. There were significant differences in weekly flower abundances among size classes (H test, P-value = 0.03) with small individuals producing fewer flowers than the large ones. Nevertheless, the number of pollinia removed (H test, P-value = 0.14), stigmas closed (H test, P-value = 0.31) and ovaries swelled (H test, P-value = 0.19) did not differ significantly. There was a significant relationship between weekly abundance of flowers and abundance of pollinators visiting flowers during the anthesis period (H test, P-value = 0.02). There was a correlation between number of pollinator visit and the number of flowers in anthesis per reproductive season (Fig. 2). The time from pollination to fruit dehiscence was between 9 and 10 weeks. The reproductive events did not differ statistically between two different coloration patterns (morphs) observed (H test, P-value > 0.05).
Flower visitors. — The most common flower diurnal visitors were herbivorous caterpillars (Lepidoptera: Geometridae), which feed on buds, ovaries, and fruits in early developmental stages. We also observed adults of two Orthoptera species: Eumastacidae and Acrididae feeding on flowers and foliage. We also saw Heliconius

cydno cydnides Staudinger (Lepidoptera: Heliconidae) visiting several flowers in a typical foraging behavior and two species of spiders, Araneidae and Thomisidae, families known to hunt pollinators. Occasionally, we observed ants eating the nectar from the lip.

We observed two pollinators species, *Bradysia* sp. (Diptera: Sciaridae) and *Mycetophila* sp. (Diptera: Mycetophilidae), both fungus gnats. The *Bradysia* sp. (Fig. 3) visited several flowers on the same plant (Fig. 4) by first landing on the synsepal, and then crossing the petals to consume the nectar on the labellum for a few seconds. Subsequently, the gnat contacted the apex of the column and probably causing the pollinarium to adhere to the ventral section of the thorax. Visits of *Bradysia* sp. were very brief (10 seconds) and were only observed on two occasions, in one of them, the individual had two pollinaria attached.

The *Mycetophila* sp. (Fig. 5) was presumably attracted by the fungus-like floral fragrance. The gnats landed on the flower (synsepal, lip, petals, or column) and began a series of wing and thorax movements while lifting the hind legs (Fig. 6), and sometimes moving the abdomen as if to oviposit. Probably pollinaria adhere to the ventral part of the thorax when individuals court and mate. Individuals stayed on the flower for a long time (sometimes overnight). We often observed many individuals of *Mycetophila* sp. visiting the flower at the same time (10 individuals), observed 47 individuals in total. On several occasions we observed mating among the *Mycetophila* sp. but we did not observe pollination events. Nevertheless, indirect evidence of their

FIGURE 4. Visit sequences of *Bradysia* sp. in *Pleurothallis marthae* flowers. a — *Bradysia* sp. arriving to the petals and facing to the labellum; b — *Bradysia* sp. consuming nectar from the labellum; c — *Bradysia* sp. crossing by the column and climbing by the lower part of the dorsal sepal; d — *Bradysia* sp. crossing by the lower part of the dorsal sepal to exit the flower.

FIGURE 5. *Mycetophila* sp. with pollinaria of *Pleurothallis marthae*. a — *Mycetophila* sp. in a flower of *Pleurothallis marthae* with a pollinarium in the ventral part of the thorax. b — Close up of *Mycetophila* sp. and pollinarium. The pollinarium was detached of the insect during the specimen manipulation.

pollination activity was obtained from the collection from the field of these diptera species with *P. marthae* pollinarium attached.

Osmophore detection. — Osmophores were detected in the epidermis of synsepals, dorsal sepal and petals, with an obvious presence in scattered spots located mainly at the edges of the structure (Fig. 7). The petal tips had glandular trichomes.

Reproductive system. — Our hand-pollination experiments indicated that *Pleurothallis marthae* is self-compatible. Self-pollination treatment within a flower produced 60% fruit set; pollination between flowers of the same clump produced 50% fruit set; cross-pollinations had 60% fruit set and natural pollination produced 40%. No significant differences were observed between the different treatments (H test, P value> 0,05).

Figure 6. Display of *Mycetophila* sp. in *Pleurothallis marthae* flowers.

Discussion. Base on the finding of *Pleurothallis marthae* is probably pollinated by two species of fungus gnats: *Mycetophila* sp. and *Bradysia* sp. Visitors can remove pollinia and not necessarily be pollinators, nevertheless, form in which deposited pollinia, court and mate in the flowers, fungus-like odor, absence of other species through visits and biology of fungus gnat, we believe that they are pollinators. This case is similar to that found by Endara *et al.* (2010), in this work on pollination of *Dracula lafleurii* Luer & Dalström and *Dracula felix* (Luer) Luer, authors argue that the pollination process occurs when individuals of *Zygothrica* perform court and mate in the flowers.

The effectiveness of fungus gnats orchid as pollinators had been briefly documented by van der Pijl and Dodson (1966) on the pollination of *Stelis aemula* Schltr. Most recently, other orchids had been reported as fungus gnats pollinated, for example *Lepanthes glicensteinii* is pollinated by *Bradysia floribunda* in Costa Rica (Blanco & Barboza 2005), *Octomeria*

crassifolia is pollinated by *Bradysia* sp. in Brazil (Barbosa *et al.* 2009). In California, *Listera cordata* (= *Neottia cordata*; Orchidoideae) is pollinated by *Mycetophila* sp. and *Sciara* sp. (Ackerman & Mesler 1979).

Mycetophila forms distinct groups of closely related species, which are within the group usually separable only by the characters on the male terminalia. *Mycetophila* is one of the most abundant families of order Diptera encompassing more than 3000 described species, with a world-wide distribution (Gaston 1991). *Pleurothallis marthae* has flowers with two different coloration patterns (morphs). Pollination success was detected equally in both morphs, suggesting that color is not a key factor determining reproductive success, and supporting the hypothesis that both fragrances and nectar are key attracting factors. Nevertheless, we found a positive relationship between flower abundance and the number of pollinator visits. This may be because with a greater number of flowers there
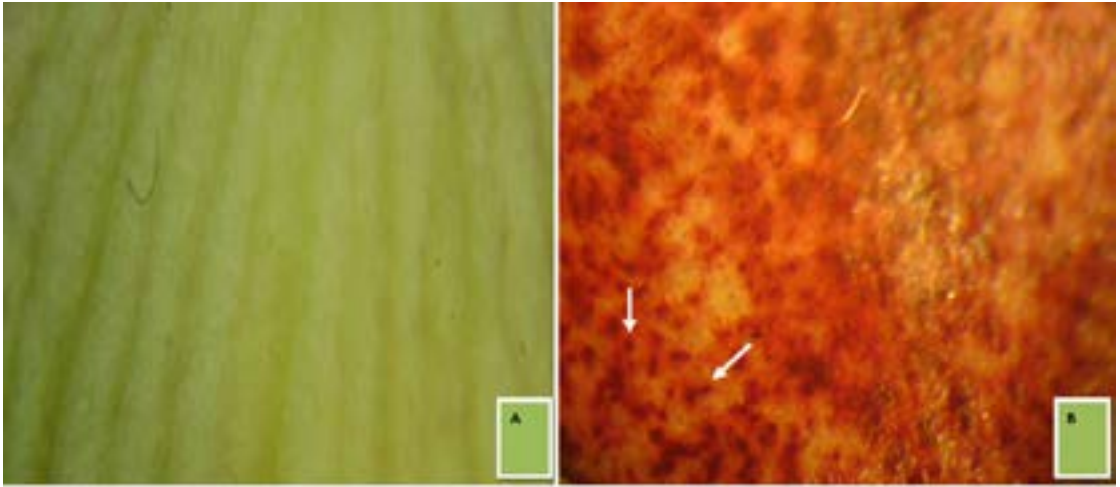
Figure 7. Portion of the dorsal sepal (a) before and (b) after staining with neutral red. Arrows shows the presence of osmophores and/or nectaries in the adaxial side of the sepal.

is likely to be an increase in the emission of aroma and possibly a greater supply of nectar, making these flowers more effective at attracting pollinators.

Pleurothallis marthae seems to mimic the smell of a fungus; this case had been reported in *Zootrophion* sp. (Orchidaceae) and coincides with the syndrome of sapromyophily (pollination syndrome of flowers producing smell of decaying flesh) proposed by Faegri & van der Pijl (1971).

Although oviposition behavior was observed by *Mycetophila* sp., we did not observe eggs or larvae under microscope, which suggests a mechanism in which the flowers attract female flies, and those females at the same time attract males (Ackerman & Mesler 1979). *Mycetophila* sp. was observed mating on the flowers on several occasions. Displaying *Mycetophila* sp. were most commonly observed on the specific parts of the flower in which we located the scent glands (osmophores), suggesting that the attraction of this species occurs by the emission of a fragrance. Additionally, floral display was also very important as plants with more open flowers had higher fitness that may be complementary.

Both pollinator species are commonly known as fungus gnats because they are almost always dependent upon fungal substrates for larval development. The *Bradysia* sp. may have been initially attracted by the smell and then by the presence of nectar, which may be a mechanism to keep the flies on flowers for longer periods of time to enhance the probability of pollination (de Melo *et al.* 2010).

Our results on the reproductive system suggest that *P. marthae* is self-compatible; this finding is compatible with the observed behavior of pollinators, whose continued presence at a single flower and at the flowers of a single plant is likely to promote *P. marthae* reproduction. This phenomenon has also been observed in other species (Mesler *et al.* 1980; Borba & Semir 2001; Singer 2001; Barbosa *et al.* 2009). While we did not conduct a pollination treatment to test for self-pollination, fruit and seed production in most self-compatible orchids are pollinator-dependent, and *P. marthae* is likely no exception (Rodríguez-Robles *et al.* 1992; Singer & Zasima 1999; Singer 2001; Singer *et al.* 2004).

The natural fruit set observed in this study was 60%. This value is higher than the ranges suggested by Neiland & Wilcock (1998) for tropical orchids, although those reported for *Listera cordata* 61-78% (Ackerman & Mesler 1979) and *Habenaria parviflora* 93.3-96.7% (Singer 2001), species pollinated by Diptera, are higher. Together, these findings suggest that Diptera pollination can be a very favorable strategy for orchids.

We present new evidence of fly pollination in the genus *Pleurothallis*. Fly pollination had already been reported for species *Lepanthes glicensteinii* Luer, *Listera cordata* (L.) R. Br., *Tolmiea menziesii* (Pursh) Torr. & A. Gray, *Asarum caudatum* Lindl., *Dracula lafleurii* Luer & Dalström and *Dracula felix* (Luer) Luer, *Specklinia pfavii* (Rchb.f.) Pupulin & Karremans

and *Specklinia spectabilis* (Ames & C.Schweinf.) Pupulin & Karremans (Mesler *et al.* 1980; Lu 1982; Goldblatt *et al.* 2004; Okuyama *et al.* 2004; Endara *et al.* 2010, Pupulin *et al.* 2012), revealing them to be very important pollination vectors that have highly specialized relationships with the plants they pollinate. As far as we know, our study is the first case suggesting fungal-like odors may be involved in attracting pollinators in *Pleurothallis* and the second for the Subtribe Pleurothallidinae (Christensen 1994). It is the first report of nocturnal pollination of the genus and species pollinated by fungus gnats. *Lepanthes* may be nocturnally pollinated too (Tremblay & Ackerman 2007). Further studies in our research group are focused in the understanding of the fragrance composition of of *P. marthae* flowers. The knowledge of this obligatory interspecific interactions is critical for the conservation management of this Colombian endemic orchid, *P. marthae*; of course, we have to include the conservation of their nocturnal fungus gnat pollinators.

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