Review Article

METHODOLOGICAL CONCERNS AND CHALLENGES IN ECOLOGICAL STUDIES WITH ORCHID BEES (HYMENOPTERA: APIDAE: EUGLOSSINA)

DESAFIOS METODOLÓGICOS EM ESTUDOS ECOLÓGICOS COM ABELHAS-DAS-ORQUÍDEAS (HYMENOPTERA: APIDAE: EUGLOSSINA)

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ABSTRACT: The Neotropical orchid bees have been the focus of several ecological studies in the last four decades, mainly due to the ease of attracting males of their species to synthetic baits that simulate floral fragrances. Moreover, orchid bees are mostly forest-dependent insects, occurring in biomes that have been rapidly wiped out and their conservation may be a matter of concern in the near future. Nevertheless, the prevalent usage of chemical attractants has hidden some problems barely discussed in the literature. Forty years after intensive studies on these bees, it is time to evaluate whether this methodology has been efficient enough to provide a clear idea of what does happen in nature. The main goal of this paper is to discuss the present status of our knowledge on orchid bees and critically evaluate the potential pitfalls of the currently used methodology, how to deal with them and the challenges for the next decades.

KEYWORDS: Chemical baits. Euglossine bees. Hexapoda. Methodology. Taxonomic impediments.

INTRODUCTION

Orchid bees (Hymenoptera: Apidae: Euglossina) are among the most conspicuous Neotropical insects, mainly due to the exuberant metallic colors of most species and their medium to large body size. Moreover, orchid-bee males have a remarkable behavior, since they actively collect floral fragrances in hundreds of plant species, especially orchids (VOGEL, 1966; DODSON et al., 1969; reviewed by DRESSLER, 1982a), which presumably are later used in courtship (see KIMSEY, 1980, ELTZ et al., 1999; BEMBÉ 2004, ELTZ; LUNAU, 2005). This unusual behavior gave ecologists a spectacular opportunity of testing several ecological hypotheses, since those fragrances could be artificially synthesized (DODSON et al., 1969) and used to easily attract orchid-bee males in their studies (e.g. KROODSMA, 1975; BRAGA, 1976; ACKERMAN, 1983, 1989; ACKERMAN et al., 1982; JANZEN et al., 1982; PEARSON; DRESSLER, 1985; POWELL; POWELL, 1987; ROUBIK; ACKERMAN, 1987; RAW, 1989; BECKER et al., 1991; ARMBRUSTER, 1993; OLIVEIRA; CAMPOS, 1995, 1996; ROUBIK, 2001; SILVA; REBÊLO, 2002; TONHASCA Jr. et al., 2002a, b, 2003; MILET-PINHEIRO; SCHLINDWEIN, 2005; NEMÉSIO; SILVEIRA, 2006a, b, 2007a, b, 2010; UEHARA-PRADO; GARÓFALO, 2006; PARRA-H; NATES-PARRA, 2007). A by-product of such studies was the improvement of our knowledge on the actual diversity of orchid-bee species and, consequently, a renewed interest in their taxonomy. More orchid-bee species have been discovered and described during the last four decades (e.g. MOURE, 1968, 1969, 1970, 1989, 1996, 1999; KIMSEY, 1977; DRESSLER, 1978, 1982b, c, d, REBÊLO; MOURE, 1996, OLIVEIRA; NEMÉSIO, 2003; ROUBIK, 2004; OLIVEIRA, 2006; RASMUSSEN; SKOV, 2006; RAMÍREZ, 2005, 2006; NEMÉSIO, 2006, 2007, 2009, 2010a, 2011c, d, e; HINOJOSA-DÍAZ; ENGEL 2007; BEMBÉ, 2008) than in the previous 200 years. Ecological studies, as a consequence, became strongly based on information retrieved from data from “populations of males”, since females are not attracted to the fragrances.

Almost half a century after the discovery of the chemical dependence of orchid-bee males on floral fragrances by VOGEL (1966) and the radical change of ecological studies about these bees, it is time to evaluate the kind and quality of data we have been gathering, the kind of information being produced and, especially, whether this methodology has been efficient enough to give us a clear idea of what does happen in nature. Are the generalizations we have been making based on male attendance to the baits correct? Are the abundances of males in orchid-bee inventories actually reflecting their relative abundances in nature? Is the use of baits good enough to carry out complete inventories of orchid-bee faunas? How far do synthesized fragrances disperse and from which distance are males attracted? Which baiting methodology is the best: traps or hand-netting? Are there non-attracted species? If yes, how can their abundances be estimated?

The main goal of this paper is to discuss the present status of our knowledge on orchid bees and critically evaluate the potential pitfalls of the currently used methodology, how to deal with them and the challenges for the next decades.

TAXONOMIC IMPEDIMENTS

Identity of species

The misuse of taxonomy is a big problem, but it is usually neglected by many ecologists (see BORTOLUS, 2008). It is impossible to consider the potential pitfalls of ecological studies with orchid bees without approaching the taxonomic impediments. As pointed out recently, "the taxonomic problems involving orchid bees—especially the two richest genera, *Euglossa* and *Eufriesea*—are a true obstacle to the development of well founded works in ecology, genetics, molecular biology, as well as in other fields of biology using these insects as models, since it is very difficult to be sure of the real identity of the species dealt with. [Moreover] There are very few specialists able to identify these bees to species level and they often have different views on the species identifications" (NEMÉSIO, 2009a: 7-8). Orchid-bee taxonomy is strongly based on male characters, unlike the prevalent usage of female characters in some other groups of bees. The reasons for this are two: (i) males became by far more abundant in entomological collections than females, due to the ease of collecting them; and (ii) males have more distinctive characters than females (or, alternatively, orchid-bee taxonomists have failed in finding reliable distinctive characters in females), particularly in the most speciose orchid-bee genus: *Euglossa*. Even among males, there have been many controversies on species identification (reviewed by NEMÉSIO, 2009a), mainly in *Euglossa*, but also in other genera, as *Exaerete* (NEMÉSIO, 2009a: 194-196). If specialists frequently disagree, of course the situation for non-specialists is still worse, since the few identification keys (e.g. MOURE, 1964; DRESSLER, 1979; KIMSEY, 1979, 1982; BONILLA-GÓMEZ; NATES-PARRA, 1992; REBÊLO; MOURE, 1996; ROUBIK; HANSON, 2004; OLIVEIRA, 2006; NEMÉSIO, 2009a, 2010a, b, 2011b) are frequently difficult to follow and many species can be misidentified.

Matching male to female

Another important taxonomic impediment is the correct use of the species names. Following the "boom" of new species being discovered after the 1970's, it has been recognized that many species were described twice, i.e., once (before the extensive use of chemical attractants) based on female specimens and again (after the use of chemical attractants) based on male specimens. As it is not easy to match males to females (see discussion in BEMBÉ, 2007 and NEMÉSIO, 2009a), the recognition of the identity of some species remains to be solved. For instance, NEMÉSIO (2009a: 92) suggested that *Euglossa pictipennis* Moure, 1943, a species for which only the female holotype is known, probably corresponds to one of the common species occurring in southeastern Brazil later described based on male specimens. This situation is not uncommon among orchid bees, and males of some species are unknown, whereas in other species the females are unknown, and these "other gender specimens" have been slowly recognized (e.g. NEMÉSIO, 2005, 2011a; LE GOFF, 2006; FERRARI; NEMÉSIO, 2011). The main problem here is that most female specimens belonging to the high species-rich genus *Euglossa* are virtually indistinguishable from their closest relatives. In spite of some attempts, both molecular (e.g. LOPEZ-URIBE; DEL LAMA, 2007) and morphological (e.g. FARIA Jr.; MELO, 2007) approaches are very scarce and deal with a very limited number of species.

Implications

The solution to these and many other problematic taxonomic impediments has more recently concerned researchers and some attempts to deal with them have been carried out (e.g. NEMÉSIO, 2009a, b). First, however, it should be emphasized how the taxonomic impediment can affect ecological studies. If specimens cannot be identified at specific levels, a great deal of important biological information is of little or no use at all. It is nonsensical to obtain biological information from non-identified organisms. Discussing ecological hypotheses based on non-identified species goes against one of the tenets of science, the possibility of repeating the experiment. If the species is unknown, how could a skeptical scientist repeat the experiment? How could he/she be sure he is using the same organism? This very problem has made some ecologists to give up using orchid bees as models in their studies. A. Tonhasca Jr. and J. L. Blackmer (pers. comm.), for example, felt very uncomfortable when unable to identify the collected specimens and, after sending them to three different specialists, realized that some bees belonging to the same "morph" returned to them with three different names!

The second problem concerning species identity has to do with the usage of specific names in comparative studies (e.g. NEMÉSIO; SILVEIRA, 2007b; SYDNEY et al., 2010). If different researchers use different names for the same species, how can one be sure of the identity of the species listed in any published work? More importantly, if used names are not reliable, how can one confidently compare orchid-bee faunas listed in works by different authors? It is easy to realize that it becomes almost impossible for any researcher trying to discuss his/her own data with those available in the literature to ask for specimens of all species listed by all previous authors to check whether the bees he/she is calling "species A" is the same "species A" listed by other authors. Comparative studies on orchid-bee biogeography and ecology become, thus, very difficult to be carried out, if not impossible at all. NEMÉSIO and SILVEIRA (2007b), for example, discussed many of these constraints and assumed that in many cases it is impossible to be sure whether species listed under the same name in different works are really the same one.

Publication of broader and more comprehensive identification keys (ROUBIK; HANSON, 2004; OLIVEIRA, 2006; NEMÉSIO, 2009a, b, 2010b, 2011b) may aid in the task of removing these taxonomic impediments, but there is still much work to do. In this respect, taxonomic works should focus on three main "fronts": (i) search for reliable characters to identify female specimens to specific level, particularly those belonging to the genus *Euglossa*; (ii) fully review the orchid bees of the Amazon, the biome with the highest number of species and the highest level of endemism (NEMÉSIO; SILVEIRA, 2007b), in order to provide a comprehensive list of species and reliable identification keys – it should be noticed that recent studies have dealt with the orchid-bee faunas of Central America (ROUBIK; HANSON, 2004) and the Brazilian Atlantic Forest (NEMÉSIO, 2009a, 2010b, 2011b, c, d, e, f, 2012); (iii) molecular research to define the status of some disputed species (reviewed by NEMÉSIO, 2009a). Only a strong and continued effort on these three fronts will promote confidence and increase stability of orchid-bee names usage.

Ecological studies based on male frequencies only

Despite the taxonomic problems mentioned above, there is an ever-growing interest for orchid bees in ecological studies. Recent suggestions that some species play a role as bioindicators (MORATO et al., 1992; MORATO, 1998), i.e., some species, such as *Eulaema nigrita* Lepeletier, 1841 and *Euglossa carolina* Nemésio, 2009 are strongly associated to disturbed areas whereas other species, such as *Euglossa marianae*, Nemésio, 2011c (until recently treated as an Atlantic population of *Euglossa analis* Westwood, 1840), are strongly associated to large and well-preserved forest patches, had a heuristic influence, leading to more detailed works to refine and test the hypothesis (e.g. TONHASCA Jr. et al., 2002a, b, 2003; MILET-PINHEIRO; SCHLIND-WEIN, 2005; NEMÉSIO; SILVEIRA, 2006b, 2007a, 2010). Regardless the hypothesis being tested, however, any ecological studies involving orchid bees are strongly based on sampling male specimens, which are attracted to chemical baits. Thus, all information on community structure (e.g. PEARSON; DRESSLER, 1985; ROUBIK; ACKERMAN, 1987; REBÊLO; GARÓFALO, 1991, 1997), ecological corridors (e.g. MOURA; SCHLINDWEIN, 2009), habitat fragmentation (MORATO et al., 1992; BEZERRA; MARTINS, 2001; TONHASCA Jr. et al., 2002; NEMÉSIO; SILVEIRA, 2007a, 2010), edge effects (e.g. NEMÉSIO; SILVEIRA, 2006b), diversity, abundance or simply inventories of orchid bees (e.g. ACKERMAN, 1983, 1989; ARMBRUSTER, 1993; SANTOS; SOFIA, 2002; AGUIAR; GAGLIA-NONE, 2008; RASMUSSEN, 2009) has been gathered from studies which methodology consisted exclusively in attracting and collecting male specimens. It is implicitly assumed that females would occur at a relatively similar abundance. But, the main question is: are males of *all* orchid bee species in a given area equally attracted to the baits? In other words, do males of *any* species in a given area have the same chance of being attracted and captured? The answer to these questions is probably "no". But we should deeply consider the attractiveness of male orchid bees to the bait scents in order to adequately answer these questions.

How responsive are orchid-bee males to synthetic fragrances?

It is well known that some species are definitely not attracted to any of the known synthetic fragrances ordinarily used in orchid-bee inventories (see MOURE, 1996; NEMÉSIO; SILVEIRA, 2004, 2006c). *Euglossa* species belonging to the subgenus *Euglossella* are typical examples of such species. Males of *Euglossa (Euglossella) mandibularis* Friese, 1899 are strongly attracted to flowers of *Solanum latiflorum* (= *Cyphomandra calycina*) in southeastern Brazil (SOARES et al., 1989), but have never been collected in any orchid-bee inventory using synthetic fragrances. Other members of *Eg. (Euglossella)* are poorly attracted to synthetic baits, such as the species *Euglossa decorata* Smith, 1874 and *Eg. viridis* (PERTY, 1833). This latter species, for example, is the main pollinator of *Cirrhaea dependens* in southeastern Brazil, but males of this species were not collected in any scent baits during the same study (see PANSARIN et al., 2006). Other examples can be found in similar studies when the main pollinator of a given orchid is not attracted to any known scent bait in the area (e.g. PANSARIN; AMARAL, 2009). Some species of *Eulaema* are also poorly attracted or not attracted to chemical baits at all. *Eulaema seabrai* Moure, 1960 and *Eulaema helvola* Moure, 2003 are endemic in eastern and central Brazil, respectively, but have never been attracted to chemical baits (NEMÉSIO; SILVEIRA, 2004, 2006c), whereas *Exaerete dentata* (Linnaeus, 1758) has a wide distribution in the Neotropics but it is rarely attracted to synthetic scents. The main question here is: if *Eulaema seabrai*, for example, is known to occur in a given area but does not attend to any bait (NEMÉSIO; SILVEIRA, 2004), how to estimate its abundance? How to deal with this fact when discussing the faunal composition of this area? Should we consider this species as "rare"? These extreme situations hide a still more complex and disturbing question: when considering only the species that are normally attracted to the scents, should we consider that *all* species respond equally to the baits? That is, if *Eulaema nigrita* and *Euglossa melanotricha* Moure, 1967, for example, are strongly attracted to cineole, and *El. nigrita* males are more collected than *Eg. melanotricha*, does it really mean that *El. nigrita* is more abundant in that given area during the sampling period? Or is it possible that a higher proportion of active *El. nigrita* males are attracted to the scents than *Eg. melanotricha*?

In spite of the tremendous recent progress in our understanding of the metabolic paths, accumulation and use of fragrances by male orchid bees (ELTZ et al., 2003, 2005a, b, 2006, 2007, 2008; BEMBÉ 2004; ZIMMERMANN et al., 2006), when considering inter-specific variation we know virtually nothing concerning how responsive are orchid-bee males to scents. Thus, any conclusion regarding the community structure and relative abundance of orchid bees based solely on relative frequencies of male attendances to chemical baits tends to be problematic.

Even males of species that are strongly attracted to synthetic baits, such as *Eulaema marcii* Nemésio, 2009 and *Euglossa imperialis* Cockerell, 1922, may avoid the baits in some circumstances. During a survey at 'Reserva Natural Vale' (municipality of Linhares, state of Espírito Santo, eastern Brazil) in April, 2009 (unpub. data), I observed a group of several male *Eg. imperialis* attracted to some natural scent produced by a trunk of a living tree just behind the area where I installed 17 different bait scents. They simply ignored the scents in favor of the trunk, although some of their "favorite" scents (cineole, methyl salicylate, eugenol and vanillin) were available. A similar episode was recorded at 'Parque Nacional do Monte Pascoal' (municipality of Porto Seguro, state of Bahia, eastern Brazil) in October 2009 (unpub. data), when at least 12 male individuals of *El. marcii* were attracted to a trunk of a living tree and ignored all the 17 scents (including their favorite benzyl acetate, β-ionone, eugenol, skatole and vanillin) installed just some five meters from the tree. These observations clearly show that not all males, even belonging to the most responsive species, are readily attracted to the baits. The questions are: how many of the active males of a given species in a given area are attracted to the baits? Does this proportion of "attracted males/total males" vary among species? In the affirmative case, our interpretation of the results obtained in male-based orchid-bee inventories may be at least somewhat biased. If some males that are just a few meters from the baits do not attend to them, it is reasonable to suppose that many other males would also fail to go to the scents. The reasons for this are not known and should be investigated. Maybe older males are less responsive to the scents than young males, although it remains as a speculation. But, for our purposes, it is enough to know that some males do not respond to the baits to launch a shadow of doubt on our data. Let me illustrate the situation with a hypothetical example: suppose that in a 50-ha area there are 500 active male *El. nigrita* and 500 active *Eg. melanotricha* at a given period, and cineole and vanillin baits are installed to attract these bees (two scents highly appreciated by both species). After a 10-hour sampling, 300 *El. nigrita* and 150 *Eg. melanotricha* are collected. All the acquired data indicate that *El. nigrita* is twice more abundant in that area than *Eg. melanotricha*, but it would be obviously incorrect. How many of us can affirm that it does not happen? If any similar situation does happen, we are failing to interpret what is actually happening in nature and reaching false conclusions about the communities of orchid bees. This simple hypothetical example does not account for more complicated and realistic possibilities. For example, *our* choice of the baits to be used (a species only attracted to methyl salicylate would not be recorded in the above example, even if it was the most abundant species at that area!). To go further in this discussion, we should thoroughly consider the baits themselves: how far do they disperse? Males from which distance are attracted? Which baits should be used? I will attempt to consider all these situations in detail below.

BAITS

How far do baits disperse?

In order to fully understand how efficient our current methodology is we should consider the efficiency of the baits themselves. In this respect, one of the most important questions is: how far do baits disperse? Dispersal of the scents is essential to our purposes. This "ability" will define how accurate is our sampling. Let us return to our previous example of a hypothetical 50-ha area inhabited by 500 active male *El. nigrita* and 500 active male *Eg. melanotricha*. Supposing that this hypothetical area is a perfect circle, if we place our cineole and vanillin baits exactly at the centre of the area, would these scents disperse to the entire area? Would a male *El. nigrita* foraging at the edge of the area perceive the scent? Were the males not attracted to the scents (in our previous hypothetical example) non-responsive? Or have they just not perceived the scents?

The current available data simply do not help us at all! We could at best make indirect inferences. For example, ARMBRUSTER (1993) made several inferences based on the within-habitat heterogeneity of orchid-bee faunas. If in a given area the scents disperse at long distances we could expect a similar faunal composition regardless of the exact location of the sampling site. But in "multiple-site studies" we see a marked difference in faunal composition among sites (e.g. ARMBRUSTER, 1993; NEMÉSIO; SILVEIRA, 2006b). It could be indicative that baits do not disperse this much and that orchid bees are not evenly distributed in a given area. Nonetheless, Nemésio and Silveira (2006b) carried out simultaneous samplings in different sites at different distances from the forest edge and those results (different faunal composition at each site) are better understood due to the differences among habitats, and not due to the dispersion of scents, since they were exposed at different sites simultaneously. Thus, our indirect evidence is not conclusive about the scent dispersion and we should consider that several factors may contribute to it, such as wind speed, air humidity and temperature, how tall are the trees and how dense is the canopy around the sampling site, and the volatility of each scent (theoretically, alcohol-based scents, such as cineole, would disperse faster than oil-based scents). All these facts, combined with Armbruster's (1993) warnings, should be considered before any conclusion on the "representativeness" of any orchid-bee inventory based on a single or few sampling sites.

Males from which distance are attracted?

The distance from which orchid-bee males are attracted to the baits is the flipside of the previous topic (how far baits disperse) and should be treated as complementary to it. If, on one hand, it is difficult to estimate the dispersive potential of each scent and, especially, how far they can reach in each particular environment, on the other hand the ability of orchid-bee males to respond to the scents is relatively well established, including the chemical and nervous paths (e.g. ELTZ et al., 1999, 2005a, b, 2008; ELTZ; LUNAU, 2005). But, in spite of male orchid bees being known as long-distance flyers (e.g. JANZEN, 1971; KROODSMA, 1975; RAW, 1989; TONHASCA Jr. et al., 2003), strongly selective concerning scents (see below) and able to detect low quantities of their preferred odors (ELTZ; LUNAU, 2005), we do not know from which distance they can detect small portions of fragrances and track a straight route to the source. Again, it will probably depend on climatic conditions, because detection will be closely related to the dispersion of scents. Nevertheless, new technologies have just become available to test this and similar questions, especially radio telemetry to track bee movements in a forested environment (WIKELSKI et al., 2010). Although unproved and expensive (and currently restricted to the larger species due to the size and weight of the transmitters), this technique may shed light upon the limits of the responsiveness of male orchid bees to baits. This could ultimately contribute to a better understanding of the distribution of orchid bees in their environment and, eventually, answer the question of how many sampling sites would be needed for a more accurate inference of the actual community composition of orchid bees in a given area.

“Bouquet” choice: how many and which baits to use?

A different approach to the “baiting problem” that was only marginally mentioned here is the “bouquet choice”. This “bouquet choice” would be best treated as “bouquet choices”, because there are two of them: the choice made by the bees themselves and the choice made by the researcher (which baits to use?). Concerning bees’ choices, it is long known that different orchid-bee species have particular preferences among fragrances (e.g. DODSON et al., 1969; JANZEN et al., 1982; ROUBIK; ACKERMAN, 1987; OLIVEIRA; CAMPOS, 1996; ELTZ et al., 2006) and that these preferences may slightly change from site to site or from season to season (ACKERMAN, 1989). Particular species may be attracted to a wide variety of scents, but I know of no species that is attracted to *all* synthetic baits used in orchid-bee inventories. For example, *El. nigrita* is attracted to several scents (e.g. cineole, skatole, *p*-cresol acetate, vanillin) but it is never attracted to eugenol. *Eulaema marcii*, on the other hand, is also attracted to many scents, but does not attend to cineole. Other species are strongly attracted to one specific bait, such as *Euglossa truncata* Rebêlo & Moure, 1996, which is strongly attracted to eugenol (e.g. REBÊLO; GARÓFALO, 1991; NEMÉSIO, 2008).

This selective preference exhibited by male orchid bees obviously affects the results of our samplings, but this problem has virtually never been discussed with the due rigor. Depending on the bouquet choice made by the researcher, the resulting inventory can vary. Is it reasonable to suppose that an inventory in which only three scents (e.g. cineole, eugenol and vanillin) are used will produce the same results of an inventory with 20 different baits at the same site? Species that are strongly or exclusively attracted to methyl salicylate, for example, would not be recorded in such inventory, even if extremely abundant at the site. A quick comparison among the tens of orchid-bee surveys easily shows that the number of used scents is extremely variable and absolutely arbitrary (e.g. BRAGA, 1976; JANZEN et al., 1982; ACKERMAN, 1983; PEARSON; DRESSLER, 1985; POWELL; POWELL, 1987; ROUBIK; ACKERMAN, 1987; BECKER et al., 1991; REBÊLO; GARÓFALO, 1991, 1997; MORATO et al., 1992; OLIVEIRA; CAMPOS ,1995; SANTOS; SOFIA, 2002; SILVA; REBÊLO, 2002; PARRA-H; NATES-PARRA, 2007; AGUIAR; GAGLIANONE, 2008; RASMUSSEN, 2009). No rationale is usually given for the specific list of used scents. The bouquet choice does not seem to be seriously considered when a research project is established and the surveys are carried out, since there is no discussion in the methodology of any published study to justify the choice. Maybe it is a matter of what scents are available at the moment. This kind of “rationale” reveals that the bouquet choice is not made with the due rigor. Except for cineole, which is included in almost all samplings, there is no standardization among studies. NEMÉSIO and SILVEIRA (2007b) have already discussed this lack of standardization and the negative consequences of it for comparative studies.

Pure or mixed?

Except for some few attempts in the 1960’s (DODSON et al., 1969), when some scents were mixed (especially to try to re-establish the actual proportion of scents found in some flowers), all ecological studies involving attraction and/or collection of orchid-bee males used pure scents as lures. This concept that pure scents are best attractants to orchid bees was strongly based on Dodson and colleagues’ (1969) influential paper and the alternative hypothesis (mixed scents could be at least as good attractants as pure scents) was never tested again, with only two exceptions (PANSARIN; AMARAL, 2009; NEMÉSIO et al., 2012).

Pure scents are usually not found in nature (but see WHITTEN et al., 1993) and definitely not found on floral sources (DRESSLER, 1982a; ELTZ et al., 2008). Thus, each orchid and other flowers which produce fragrances attractive to orchid bees present a specific composition of several scents that usually attracts only one or most commonly a few orchid-bee species (reviewed by DRESSLER, 1982a). On the other hand, the bouquet of scents collected by orchid-bee males is usually different from those offered by any specific flower, in such a way males must visit different sources to gather all the needed scents in the required proportion (which varies from species to species) (ELTZ et al., 1999, 2005a). In other words, orchid-bee males are able to perceive small fractions of the required scent, *even when mixed to other scents*. This fact led me to reconsider the idea that pure scents are always the best choice to attract orchid bees and, especially, led me to consider the possibility that some mixtures of scents could be attractive to some species that did not attend to the pure fragrances.

Recently, I carried out some experiences in forested protected areas in southern Bahia, eastern Brazil, and opted for mixing scents randomly, not trying to re-establish the actual proportion of scents found in any particular plant species (unpub. data). Thus, I mixed cineole in a 1:1 proportion to 15 other different scents and subsequently mixed each one of the other 15 scents in a 1:1 proportion to the other scents. I also tried different proportions (2:1, 3:1, 4:1) of those most successful mixtures and realized that some of these random mixtures are at least as attractive to orchid bees as pure ones. The most interesting finding was that one of the rarest species of orchid-bee, *Euglossa cyanochlora* Moure, 1996, recently proposed to be endangered (NEMÉSIO, 2009a), was first attracted and collected in a mixture of two rarely used scents (although later I collected this same species in each one of the pure components of the mixture – see NEMÉSIO et al., 2012). Thus, I here suggest that an entire field for testing mixtures of scents may be open and, maybe, some of these mixtures may be attractive to rarely collected species. The efficiency of the pure scents is not questioned. But some mixtures can improve our ability to detect some species otherwise unnoticed if only the traditional methodology (pure scents) is adopted.

Additional attention should be paid to the fact that attractiveness of pure synthetic scents and natural scents may be very different. For example, a recent study shows that tens of bees are attracted to flowers of *Cirrhaea dependens* when they just open (before sun rising), regardless air humidity, wind speed, rain and other climatic factors (PANSARIN et al. 2006). This study suggests that attractiveness of floral fragrances may be stronger to particular bee species than any pure scent, since these scents rarely attract a huge number of bees in early morning.

“Uncertainty principle”

Another implication of using synthetic scents to attract orchid-bee males has a more philosophical basis: are we studying the actual behavior and distribution of these bees or are we “forcing” them to be where they would not be if we had not placed the synthetic baits in the field? There is at least one record of a species being attracted to an environment where it usually does not occur looking for synthetic baits: one male *Eufriesea nigrohirta* (Friese, 1899), a species endemic in the “campos rupestres” (rocky fields above 1,000 m a.s.l. in southeastern Brazil), was attracted to a methyl-cinnamate bait in a small patch of forest (NEMÉSIO; SILVEIRA, 2007a). Would this bee enter the forest if we had not placed this pure scent there? Do orchid bees frequently occur at the understory of forests or are they there only because we place our baits slightly over the soil? All these questions are somewhat analogous to Heisenberg uncertainty principle (HEISENBERG, 1927) in physics, of course only in the philosophical sense. At the very moment we place our baits in the field we probably interfere with the movements and distribution of male orchid bees, and we may artificially “force” bees to be in areas where they would not be naturally. It may be the situation in large forest patches, when species clearly intolerant to open environments found at the edges are attracted to these sites searching for the synthetic baits placed there (see NEMÉSIO; SILVEIRA, 2006b). As an “uncertainty principle”, it becomes virtually impossible to test the hypothesis, but it is important to call attention to the fact that we are introducing scents with such a high level of pureness that it is possible that our results do not represent what would actually occur in nature.

“Contamination” and how to avoid it

A basic problem, especially for beginners in orchid-bee studies, is the possibility of “contamination”. By contamination I mean the accidental mixture of scents and the consequent impossibility of knowing what scent actually attracted the bee. Contamination can occur at several stages of field work, but two circumstances are the most common: (i) when manipulating the scent vials, the researcher’s hands or gloves may be contaminated with one scent and, by manipulating the subsequent bait (the string or the cotton piece where the scent will be applied) there would be a mixture of scents; (ii) when inadequately using the insect net to capture the bees. It is common for the collector to move the net towards the bait (where the bee supposedly is) in a fast movement to capture the bee. Often, the net will touch the bait and this, in turn, will impregnate the net with the scent. After some accidental impregnations from different scents, the bees will be attracted to the net itself and not to the pure scents and the researcher will not be able to know which specific scent a bee was attracted to. There is a simple tip to avoid this last kind of contamination. I long ago realized that orchid-bee males, a few seconds after touching the scent and starting to collect it, become less shy and usually allow that the collector gently position the net below them. If the researcher waits until a male makes one of its several small flights while collecting the scent, in a fast upward movement it is possible to collect the bee without touching the bait. It is still very easy to conduct the male to a safe distance from the bait, because after the net is positioned below the male, it takes the net as its point of reference and tends to follow the net. This is the best moment to collect the bee (with a fast upward movement) without risking contamination of the insect net. Of course the situation becomes more complicated if many males approach the bait at the same time, but with some practice it is possible to collect all of them with this method.

Baiting methodology: traps or hand netting?

Baiting methodology is the only topic in the present discussion that has received some kind of attention (BECKER et al., 1991; MORATO, 1998; NEMÉSIO; MORATO, 2004, 2006; NEMÉSIO; SILVEIRA, 2007b) and even some empiric field works (NEMÉSIO; MORATO, 2004, 2006; MATTOZO et al., 2011). Our best data suggest that hand netting is more efficient than trapping, at least in those areas, as in the Amazon, where very large and very small orchid-bee species occur in sympatry. It means that the entrance hole in the traps should be large enough to allow the big bees (as the largest species of *Eulaema* and *Exaerete*) to enter the trap. Of course, if the hole is wide enough to allow *El. bombiformis* (Packard, 1869) to enter, it will be also big enough to allow the small *Euglossa* to escape. This matter was thoroughly discussed by NEMÉSIO and MORATO (2004, 2006) and empirical data showed that there is a strong bias when hand netting and trapping are compared: *Eulaema* spp. are proportionately much more abundant in trap collections than in hand net collections. According to field data and observations by NEMÉSIO and MORATO (2004, 2006) in the Amazon, as well as recent observations in the Atlantic Forest of southern Bahia (unpub. data), this is a natural consequence of the high number of escapes of male *Euglossa* spp. Moreover, the high elapsed time spent by male *Eulaema* spp. trying to enter the trap (and, thus, blocking the trap and obstructing the entrance for the small *Euglossa*) contributes to this bias. Besides this difference in the proportion of *Eulaema* and *Euglossa* species between both methods, another disadvantage of using traps is the lower number of captured bees when compared to hand netting (NEMÉSIO; MORATO, 2004, 2006; MATTOZO et al., 2011). The whole discussion about baiting methodology has only one goal – allow the comprehension of what happens in nature. The use of bait traps clearly introduces a bias, since not all bees attracted are collected and many trapped bees manage to escape, providing results that are very different from what occurred in nature at the sampling site. As discussed above, there can be many problems in attracting male orchid bees with synthetic scents (e.g. some species are not responsive, we do not know how many males of each species are responsive) but hand netting, at least, has a higher chance of telling us how many and which species were attracted to the baits, something that baited traps often fail to do.

Hand netting: efficiency of the collector. How many bees do escape?

If hand netting has a higher potential of revealing how many and which species are attracted to baits, the efficiency of the collector must be taken into account when we evaluate whether that potential was achieved. Orchid bees are fast, shy insects, and not easy to collect with an insect net by a non-experienced collector. Even a highly trained collector loses at least some specimens in every sampling. Nevertheless, this information is rarely present in literature (e.g. ROUBIK; ACKERMAN, 1987). Researchers, including myself, rarely take notes of how many bees escaped during a sampling. Thus, in spite of being more efficient than bait trapping, hand netting is far from a 100% reliable method and there are no estimates of a possible bias (if one species escapes more often than others). My own observations revealed that *Eulaema marcii* escapes much more frequently than *El. nigrita*, for example. But I have never quantified these escapes to offer an accurate and objective discussion on the subject and I do not know of any study which has presented such data. Different researchers have different abilities for collecting with insect nets and these differences have never been quantified. These differences certainly are not the main problem with the baiting methodology, but it is another point that can contribute to increase the bias. Contrary to other problematic questions raised in this paper, the solution to this “problem” is quite easy: it will be enough if from now on researchers include in their results section the number of bees that visited the baits but were not collected, preferably informing the genera of the bees and, whenever possible, the species.

HOW TO ESTIMATE THE ABUNDANCE OF NON-ATTRACTED SPECIES?

Despite all problems discussed above regarding the use of synthetic baits and their actual attractiveness to male orchid bees, this methodology makes it possible to survey a considerable number of species in any given area in the Neotropical region. The same is not true for those species poorly or no attracted to the scents, as mentioned above. How, then, to estimate the abundance of these latter species or even record their presence? It is a difficult matter and I see only two options, both much more complicated than the use of baits as lures.

Abundance estimated through females

The first option is to study the orchid-bee community through the presence and abundance of females. There are two problems with this method: the first and most serious one is that females of most *Euglossa* species cannot be identified at species level, as discussed in the "Taxonomic impediments" section. The second problem is that orchid bees are mostly forest insects. In forests, flowers are usually on the canopy and bee collection on flowers is very difficult in such environments. If taxonomists manage to identify female *Euglossa* spp. at species level, however, samplings based on females collected at the edge of the forest may be useful. At the edge there are plenty of flowers at the lower strata of vegetation and such a sampling can be carried out. Studies based on males (e.g. MORATO et al., 1992; NEMÉSIO; SILVEIRA, 2006b) show that there is a marked change in the orchid-bee fauna from the edge to the interior of the forest, and sampling females on the canopy at the sites in the interior of the forest is not a feasible option. But a comparison between male and female faunas at the edge may shed some light upon this question.

Abundance estimated through nests

The alternative possibility is the study of the orchid-bee fauna through nests. In nests, both males and females can be collected and male identification is warranted in most cases. Estimating bee diversity through nests is not new. For some taxa of eusocial bees, as stingless bees (Meliponina), there are studies focusing on nests to sample bee faunas (e.g. SIQUEIRA et al., 2007; SERRA et al., 2009). Nests of orchid bees, however, are usually not as conspicuous as those of stingless bees. Maybe for this reason, only about 20% of orchid-bee species have their nests known and described (AUGUSTO; GARÓFALO, 2007). Nevertheless, the methodology of trap nests has been successfully used to attract orchid-bee females which nest in these traps (usually pieces of bamboo) (GARÓFALO et al., 1993). The success of colonization of trap nests by female orchid bees seems to be low, but it should be considered that there are few researchers working on orchid-bee nesting biology (e.g. ROBERTS; DODSON, 1967; YOUNG, 1985; GARÓFALO, 1985, 1992; GARÓFALO et al., 1998; SOUCY et al., 2003; AUGUSTO; GARÓFALO, 2004). Nevertheless, some interesting data are available that are complementary to the previous discussion on the representativeness of collections using chemical baits. For example, *Euglossa aratingae* Nemésio 2009a is a species rarely attracted to baits, but nests of this species are among the most commonly found and this species is also one of the most frequently found in trap nests (AUGUSTO; GARÓFALO, 2004, treated as *Euglossa townsendi* Cockerell, 1904). It is important to notice that the study by AUGUSTO and GARÓFALO (2004) was conducted in the same macro-region where REBÊLO and GARÓFALO (1991, 1997, also treated as *Eg. townsendi*) carried out several samplings using chemical baits. It seems that males of this species are poorly attracted to the scents and their frequencies in male samplings do not reflect the abundance of this species in nature. Searching for nests and use of trap nests seem to be reasonable alternatives to bait sampling when we try to investigate the relative abundance of orchid bee species. At least, it would be an important complementary methodology.

IMPACT OF COLLECTIONS: HOW MUCH TO COLLECT?

Most orchid bees are forest-dependent organisms (e.g. DRESSLER, 1982a; ROUBIK; HANSON, 2004). As Neotropical forests are under heavy anthropogenic pressure, especially in Central America and in the Brazilian Atlantic Forest, with the resulting loss of the original cover and consequent fragmentation, conservation of orchid bees is and will be more and more an important issue. Comments on the rarity of some orchid-bee species have already been made by Nemésio and Silveira (2004) and Nemésio (2011c, f), but Nemésio (2009a) was the first one to try to assess the conservation status of several orchid bees (all Atlantic Forest species) using the IUCN criteria (IUCN 2001) and suggested that some species should be included in official red lists of threatened species (e.g. *Eg. cyanochlora*). As deforestation continues to take place, it is possible that the most sensitive species will continue to decline and orchid-bee researchers should consider these constraints when using these insects in ecological studies that involve collection and sacrifice of specimens. The collection of orchid bees, especially *Euglossa* spp., is almost inevitable because most species of this genus cannot be readily identified in the field. Moreover, predatory collections may also threaten the plant species that depend on these bees to be pollinated. This situation may result in a difficult decision for the researcher, especially in those areas where several inventories were already carried out: to collect or not? In affirmative case, how much to collect?

There are no studies focusing on the impact of collections on populations of orchid bees. Typical procedures consist in one to two samplings monthly during 12 consecutive months. Very few studies were carried out for more than one year (e.g. ROUBIK; ACKERMAN; 1987) and it is difficult to draw any conclusions from them. The classical work by ROUBIK (2001) gives us a concise idea of the fluctuations in orchid bee populations during a long series (21 years), but no systematic collections were carried out and it is impossible to evaluate the impact of collections from that study. An unpublished study carried out at a forest patch in Belo Horizonte city, state of Minas Gerais, Brazil, during four alternate years, however, suggest some tendencies. This study was conducted every other year from 1997 to 2004, sampling orchid bees (collecting and sacrificing) exactly at the same site, using the same five chemical baits (cineole, benzyl acetate, eugenol, methyl-cinnamate and vanillin), and in the same time span (from 10:00h to 16:00h). Although there was a marked fluctuation, especially among the three first years (see Figure 1), there was not a continuous decline and the relative abundances of each species remained reasonably constant over the entire study period (unpub. data). On the contrary, the third and fourth years recorded an increase in abundance when compared to the second year (Figure 1). This result suggests that the typical procedures mentioned above do not affect the community structure or lead specific populations to decline, but it should be regarded with great care because the sampling was carried out in a forest patch immersed in an urban matrix, i.e., most species occurring there are reasonably tolerant to anthropogenic disturbance and immigration from other forest patches can account for the relative stability of the populations. More studies are urgently needed to assess the actual impact of collections on populations of orchid bees.

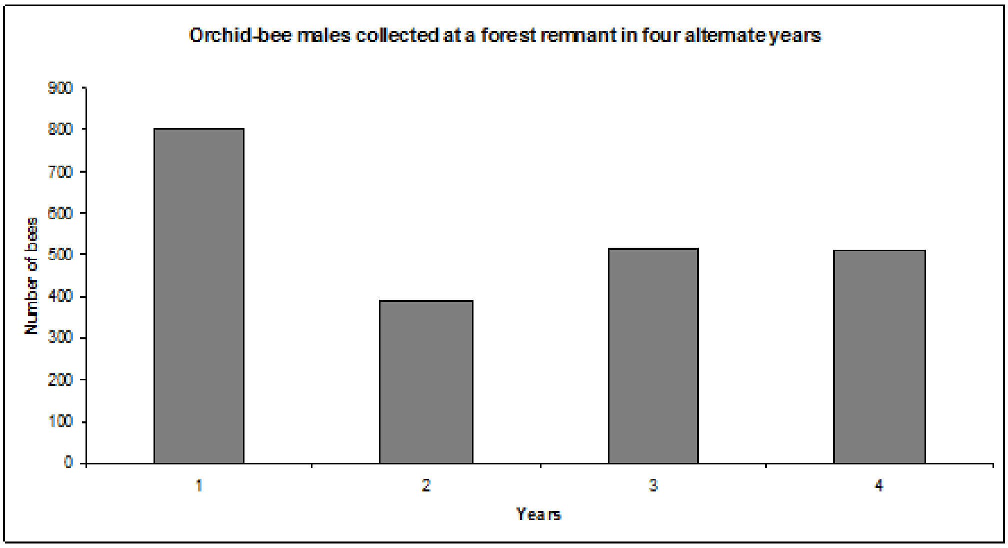

Figure 1. Orchid-bee males collected at Parque das Mangabeiras, municipality of Belo Horizonte, state of Minas Gerais, Brazil, from April, 1997 to March, 2004. Year 1: April, 1997 to March, 1998; Year 2: April, 1999 to March, 2000; Year 3: April, 2001 to March, 2002; Year 4: April, 2003 to March, 2004.

FINAL REMARKS

Field studies with orchid bees have been strongly based on the traditional methodology of baiting male specimens. Nevertheless, there is enough evidence that this methodology is not enough to provide a thorough view of the complexity of orchid bee communities. Additional methods (trap nesting, searching for nests, and collection on flowers) should be more and more part of orchid bee inventory strategies. Taxonomic work should urgently focus on searching reliable characters to identify females of the speciose genus *Euglossa*. Authors should be more descriptive when recording their results. As mentioned before, the number of specimens not collected due to collector's failure should be recorded. On a different perspective, future studies should focus on the mechanisms of attraction of bees by natural and synthetic fragrances: how far do they disperse, is there a difference between natural flower fragrances and synthetic pure ones? Why do some flowers attract so many specimens even in complete darkness, even under rain, whereas scent baits are more attractive in dry and hot days, usually in the mid of the morning? These and many more questions raised previously in this text remain to be answered and should be the next frontier in orchid bee studies.

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RESUMO: As abelhas-das-orquídeas têm sido o foco de diversos estudos ecológicos nas últimas quarto décadas, especialmente devido à facilidade de se atrair machos dessas abelhas à iscas sintéticas que simulam o odor de fragrâncias florais. Além disso, as abelhas-das-orquídeas são insetos dependents de áreas florestadas, ocorrendo em biomas que têm sido rapidamente desmatados e sua conservação pode ser um tema relevante no futuro próximo. Não obstante, a metodologia prevalente em inventários dessas abelhas esconde alguns problemas raramente discutidos na literatura. Quarenta anos após intensivos trabalhos com essas abelhas, é o momento de se avaliar se essa metodologia tem sido eficiente o suficiente para nos fornecer uma ideia precisa do que ocorre na natureza. O principal objetivo desse artigo é discutir o estágio atual de nosso conhecimento sobre as abelhas-das-orquídeas e avaliar os problemas potenciais da metodologia correntemente utilizada, como lidar com esses problemas e os desafios para as próximas décadas.

PALAVRAS-CHAVE: Abelhas euglossinas. Hexapoda. Impedimentos Taxonômicos. Iscas Químicas. Metodologia. Taxonomic impediments.

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