

Invasive bees promote high reproductive success in Andean orchids



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ARTICLE INFO

Article history:

Received 29 November 2013

Received in revised form 3 April 2014

Accepted 7 April 2014

Keywords:

Bombus
Honeybees
Invasive species
Mutualism
Orchidaceae
Patagonia

ABSTRACT

Most non-autogamous orchids rely on either a single, or on a small number of pollinators to set fruit. The nectar-rewarding orchid *Brachystele unilateralis* and the nectarless and deceit-pollinated *Chloraea virescens* are restricted to the Southern Andes, where a single native and endemic bumblebee (*Bombus dahlbomii*) occurred until the introduction of honeybees and highly invasive European bumblebees. Here, we describe the floral features, breeding system, pollinator activity and fruiting success of these orchid species. Both are self-compatible but pollinator-dependent, i.e., unable to set fruit and seed in the absence of pollinators. Field observations revealed that most of their current reproductive success is due to the introduced *Bombus terrestris*, *Bombus ruderatus* and *Apis mellifera* bees. The only native pollinator recorded was *B. dahlbomii*, but this bee was rarely observed due to its steady decline since the introduction of the alien *Bombus* species. The observed natural fruiting success per inflorescence in both studied species proved to be remarkably high by orchid standards: 83% and 66%, respectively. These results suggest that sexual reproduction in these orchids is not necessarily threatened by the decline of their native pollinator since pollination is successfully achieved by introduced bees. This provides a plausible better outlook for these and other regional, bumblebee-pollinated orchids and raises caution on eventual conservation policies involving the management of these introduced bee species. To our knowledge, this is the first report of native orchid pollinators being effectively replaced by invasive bumblebees and, furthermore, the first description of the pollination biology of a species of *Brachystele*.

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1. Introduction

Biological invasions constitute a major threat to global biodiversity by establishing exotic species within native communities and thus changing the structure and function of ecosystems by means of new, direct and indirect interactions between species (Parker et al., 1999; Traveset and Richardson, 2006). Recently, it has been asserted that the incorporation of invasive species within native pollination networks remarkably modifies the architecture of the latter: the invaders tend to monopolize the majority of web interactions by becoming “super-hubs”, while connectivity between native species diminishes (Aizen et al., 2008; Santos et al., 2012; Traveset et al., 2013). These rearrangements increase the asymmetry between interactions and their nestedness which is likely to

result in networks that, whilst more robust against the loss of certain species (Aizen et al., 2008; Traveset and Richardson, 2011; Traveset et al., 2013), are more susceptible to the cascading effects that follow disturbances (Traveset et al., 2013).

The potential undesirable effects of invasive bees on their native counterparts range from competition for resources and nesting sites to the transmission of pathogens, reproductive disruption and hybridization (Goulson, 2003, 2010; Stout and Morales, 2009). The effects on native plants vary from being detrimental to beneficial (Traveset and Richardson, 2006). Moreover, native plants that depend on one or two pollinators are likely to be less buffered from these effects than plants visited by several native pollinators (Traveset and Richardson, 2006). These negative effects are associated with foraging behaviour and morphological mismatches between the bee and the visited flowers which, in turn, result in an inefficient pollen removal/deposition rate and ultimately to reduced seed set (Gross and Mackay, 1998; Roubik, 1996 cited in Dick et al., 2003; Whelan et al., 2009). Furthermore, a different pattern of pollen transport to that of native pollinators may alter the genetic structure of the plant population (Dick, 2001; Dick et al., 2003; Dupont et al., 2004; England and Beynon, 2001;

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Whelan et al., 2009). In self-incompatible or dioecious plants, this can result in reduced seed set (Hansen et al., 2002; Kenta et al., 2007). Introduced pollinators may also promote beneficial interactions. For example, alien pollinators can increase plant fitness by increasing pollen transfer between flowers (Cayuela et al., 2011; Gross, 2001; Paton, 2000; Madjidian et al., 2008; Sun et al., 2012). They can also ensure cross-pollination by long-distance pollen flow (Dick, 2001; Ottewell et al., 2009). Finally, invasive bees may successfully provide essential ecosystem services in degraded or fragmented habitats where native pollinators may have been lost or are in decline (Dick, 2001; Downing and Liu, 2012; Goulson, 2003; Gross, 2001; Ottewell et al., 2009; Sun et al., 2012).

In the temperate forest of southern South America dwells a single endemic species of bumblebee, *Bombus dahlbomii* Guérin-Méneville (Abrahamovich and Díaz, 2001), which is considered a keystone species (Bond, 1994) as it is an extremely generalist pollinator upon which many species depend on for their reproductive success (Aizen et al., 2002, 2011; Vázquez and Simberloff, 2003). The introduction of exotic bees to these temperate forests began with *Apis mellifera* L. for apiculture (Pérez Rosales, 1859) followed in 1984 by *Bombus ruderatus* (Fabricius) for the pollination of red clover, again to be followed by the most recent introduction, *Bombus terrestris* (L.) in 1998, for the pollination of greenhouse tomatoes (Torretta et al., 2006 and references therein). The invasive bumblebees expanded their range while *B. dahlbomii* simultaneously experienced a geographic retraction in its range and a population decline (Arbetman et al., 2012; Madjidian et al., 2008; Montalva et al., 2011; Morales, 2007; Morales et al., 2013). These three invasive pollinators represent 15% of the bee species introduced outside their native range for intentional release and trade in colonies (adapted from Stout and Morales, 2009). Their invasive capacity seems to be related to their highly plastic behaviour, their efficient search for resources, their exploitation of a wide niche and their indiscriminate and active flower foraging (Aizen et al., 2008; Goulson, 2003; Ings et al., 2006; Morales, 2007).

Non-autogamous orchids, in general, depend heavily on a few pollinator species for their reproductive success (Neiland and Wilcock, 1998; Pemberton, 2010; Schiestl and Schlüter, 2009; Tremblay, 1992). This implies a very tight and specific plant-pollinator relationship, where reproductive success is limited by pollinator availability and abundance (Tremblay et al., 2005). As a result, fruit and seed-set in these plants are intimately and directly associated with the conservation of their pollinators (Wilcock and Neiland, 2002). On this account and considering that most orchids present their pollen packed in pollinaria that are easily observed on the animal pollen-vectors, these plants become a feasible model to study the effects of introduced pollinators.

As part of our studies on the pollination strategies and breeding systems of native terrestrial orchids, we focused on two late-blooming sympatric species, *Chloraea virescens* (Willd.) Lindl. and *Brachystele unilateralis* (Poir.) Schltr., from Patagonian forests with *a priori* different pollination strategies in a location where we confirmed the presence of these three alien pollinators and where *B. dahlbomii* is scarce and seldom seen. The main questions that this study addresses are: (a) Are these orchids pollinator-dependent? (b) Is the decline of the keystone species *B. dahlbomii* threatening the reproductive success of the studied orchid populations?; (c) Did novel pollination interactions arise with the arrival of invasive species?

2. Materials and methods

2.1. Study site

Studies were performed on populations located on the west slope of Mt. Currumahuida, in the municipality of Lago Puelo in

Chubut Province, Argentina (42.09°S; 71.59°W). This site is located in the oriental side of the Andes and represents the transition between the Sub-Antarctic and Patagonic Provinces from the Antarctic and Altoandina Phytogeographical Domains, respectively (Cabrera, 1971). This mountain reaches up to 1300 m.a.s.l. and constitutes an ecotone where Patagonic cedar forests (*Austrocedrus chilensis* (D. Don) Pic.Serm. & Bizzarri, Cupressaceae), shrublands of *Fabiana imbricata* Ruiz & Pav. (Solanaceae), *Colletia* spp. (Rhamnaceae) and *Mulinum spinosum* (Cav.) Pers. (Apiaceae), and *Nothofagus dombeysi* (Mirb.) Oerst. (Nothofagaceae) forests coexist. A long history of disturbances such as intentional fires and logging has created a mosaic of different successional states. The climate corresponds to the category Csb, based on the classification proposed by Köppen (Kottek et al., 2006), and this is characterized by dry warm summers and cool winters with rain and snowfall. The average annual rainfall is c. 1300 mm and the annual average temperature is approximately 11.0 °C (ICE-PNL, pers. com.).

2.2. Species studied

C. virescens and *B. unilateralis* are endemic to the southern Andes. *C. virescens* grows in Chile from the Vth to the Xth administrative region (33–39° south) and in Argentina in the provinces of Neuquén, Rio Negro and Chubut (39–44° south) (Correa, 1969; Schinini et al., 2008). *B. unilateralis* dwells in Chile from the IVth to the Xth administrative region (32–44° south) (Novoa et al., 2006), and in Argentina is found only in a small area near the 42nd parallel (Correa, 1955) associated with an Andean pass. They flower in mid-late local summer and produce a spike reaching up to 80 cm and 50 cm for *C. virescens* and *B. unilateralis*, respectively. From autumn until summer, they are found as rosettes of basal leaves that wilt and dry during anthesis.

Their conservation status still remains to be assessed, both on a global scale (IUCN, 2014) and on a national level (MMA, 2014; Planear, 2014). However, we believe that, owing to the restricted distribution of *B. unilateralis* in Argentina, this species should be included in the IVth category of the Argentinian list of threatened plants, based on the criteria set down therein (Planear, 2014). This IVth category represents those species considered by the IUCN to be vulnerable (VU) at national level.

2.3. Floral features and nectar properties

Fresh flowers were used to record and measure flower features (Table 1). Nectar parameters were measured on two potted *B. unilateralis* plants around mid-day, when pollinator activity was greatest in the field. Nectar volume was measured by means of a P2 micropipette and total sugar concentration was measured and adjusted relative to temperature by means of a Reichert AR2000 digital refractometer.

Morphological features were photographed using a DSLR camera and flash. Plant vouchers were deposited at the BA Herbarium of the Museo Argentino de Ciencias Naturales (MACN) under the following accession numbers: *C. virescens* (A. Sanguinetti 89) and *B. unilateralis* (A. Sanguinetti 91). Throughout this paper we follow the orchid morphology concepts of Dressler (1993). Specific morphological concepts for Chloraeinae orchids follow Sanguinetti et al. (2012).

2.4. Breeding system

Breeding system experiments were performed in the field by bagging inflorescences in order to exclude natural pollinators. Four treatments were randomly applied to each of these inflorescences: intact flowers (control); emasculation; manual self-pollination (flowers pollinated with its own pollen); and manual cross-pollination (flowers pollinated with the pollen of another conspecific

Table 1
Floral traits of *Chloraea virescens* and *Brachystele unilateralis*.^a

Floral traits	<i>Chloraea virescens</i>	<i>Brachystele unilateralis</i>
Flowers/inflorescence	10.9 ± 4.3 (3–21; 1058; 97)	41.4 ± 9.5 (26–58; 662; 16)
Floral lifespan (days)	18.8 ± 3.8 (11–25; 26; 9) ^b	16.6 ± 3.7 (9–24; 37; 10)
Lifespan emasculated (days)	19.7 ± 5.1 (9–32; 25; 9) ^b	–
Male phase (days)	–	10.8 ± 1.2 (10–13; 6; 2)
Pollinarium size (mm)	4.5 × 3	2.5 × 1.5
Nectar volume (μL)	–	0.76 ± 0.59 (0.15–2.05; 20; 2)
Sugar concentration (%)	–	58.83 ± 7.40 (46.90–69.50; 20; 2)
Scent	Slightly rose-like	Sweet and slightly lemon-like

^a Numerical data presented as: Mean ± Standard Deviation (minimum–maximum value; number of flowers; number of individuals).

^b Both treatments were not statistically different following a two-tailed *t*-test ($t = 0.69$; $p = 0.49$).

individual) (Table 2). Emasculatation was performed on *C. virescens* by removing the whole anther and on *B. unilateralis* by removing the pollinarium. When appropriate, treatments yielding fruit were compared using a χ^2 test (Table 2). Intact and emasculated flowers of *C. virescens* were used to record the flower lifespan for each treatment, observations being made every 2 days. Both treatments were compared using a two-tail *t*-student test (Table 1). Intact flowers of *B. unilateralis* were used to record flower lifespan, observations being made every 2 days.

2.5. Pollination

The pollination biology of both species was studied in the field during the flowering seasons of 2012 and 2013, from January to March (Table 3). The total number of observation hours for *C. virescens* and *B. unilateralis* were 111 h and 117 h, respectively (Table 3). Since both species emitted noticeable fragrances only during the day, observations were all made then, commencing at 8:30 h and ending at 20:30 h (dusk). In this contribution, the term “pollinators” is restricted to those visiting insects which were able to dislodge and deposit pollinaria during visitation events. Insects unable to dislodge and deposit pollinaria during visitation are referred to as “visitors”. Pollinator behaviour was documented for both species in the field following the methodology described by Sanguinetti et al. (2012). A video record made it possible to obtain a more objective interpretation of pollinator behaviour and to monitor accurately the number of flowers visited by the bees and the time spent at each flower (Table 4). Individual insects carrying pollinaria were collected and sacrificed for taxonomic identification. These insect vouchers were deposited in the entomological collection of MACN.

2.6. Fruiting success and pollination efficiency

In order to assess the effectiveness of pollination, the fruiting success (number of fruit divided by the number of flowers produced per inflorescence) was calculated for each species at the end of each flowering period. During 2012, 65 inflorescences of *C. virescens* (totaling 883 flowers) and 10 inflorescences of

B. unilateralis (totaling 372 flowers) were counted. During 2013, 97 inflorescences of *C. virescens* (totaling 1050) flowers were counted (Table 5). It was not possible to record the fruiting success of *B. unilateralis* for 2013 due to the abnormal development of most of the flowers observed at the studied population (the viscidia of most flowers were not attached to the pollinia, as is the case in normally-developed flowers; we speculate that this abnormal flower development could be attributed to the unusual dryness and high temperatures experienced during that particular summer). Fruiting success for *C. virescens* was compared statistically for both years using a non-parametric two-coiled Mann–Whitney *U* test (Table 5), since the data did not conform to a normal distribution based on the Shapiro–Wilk test (results not shown).

A male efficiency factor was used to estimate pollinator efficiency at pollination per pollinarium removed (percentage of fruiting flowers divided by the percentage of flowers acting as pollen donors; modified from Nilsson et al., 1992) for both species in 2012 and for *C. virescens* in 2013 (Table 5).

3. Results

3.1. Floral features

Only floral morphological features relevant to the pollination process will be presented here. Readers interested in further details relating to both orchid species studied are referred to the works of Correa (1955, 1969). *C. virescens* has large, white and showy flowers, marked green, and grouped on a slender terminal spike (Fig. 1A and B). The adaxial surface of the labellum bears green falcate to lanceolate projections. Since the labellum is hinged (articulated) at the base of the column, it becomes depressed as insects enter the floral cavity (see Section 3.3). Petals mostly hide the column, restricting pollinator access to the interior of the flower (Fig. 1B and C). The column is elongate, slightly curved and has two openings (*fossae*) at its base that extend to half the length of the ovary, but these do not contain free nectar (Fig. 1F, Supplementary material 1). The stigma is subterminal and triangular, with a glandular, slightly differentiated, distal rostellum (Fig. 1C and E). The anther is terminal and produces a large

Table 2
Breeding system experiments. Fruiting success (%) in *C. virescens* and *B. unilateralis* for intact (Control), emasculated (Emasculatation), hand self-pollinated (Self-pollination) and hand cross-pollinated (Cross-pollination) flowers.^a

Orchid species	<i>N</i> [†]	Control	Emasculatation	Self-pollination	Cross-pollination
<i>C. virescens</i>	15	0% (0/46)	0% (0/46)	100% (47/47)	100% (49/49)
<i>B. unilateralis</i>	8	0% (0/85)	0% (0/70)	88.0% (66/75) ^b	89.2% (66/74) ^b

[†] Number of inflorescences used in the experiments.

^a Numbers in parentheses represent the number of fruit obtained over the number of flowers used in each treatment.

^b Differences in the fruiting success of *B. unilateralis* were not statistically significant following either self- or cross-pollination according to the chi-square test ($\chi^2 = 0.052$; $p > 0.05$).

Table 3Observation period and number of hours spent each season observing *C. virescens* and *B. unilateralis*.

Species	Year	Observation days	Observation period	Observation hours	Observed population sizes (inflorescences)
<i>C. virescens</i>	2012	26, 27, 31 January and 1, 2, 5, 7, 9–11 February	10:45 to 19:00 h	58	3
	2013	12–15, 17, 20 January	8:00 to 17:00 h	53.5	21
<i>B. unilateralis</i>	2012	27, 31 January; 1, 2, 5, 7, 9–11, 18–20, 22, 23, 27, 29 February and 2 March	10:00 to 20:30 h	95	3
	2013	5, 7, 12–14 February	10:00 to 18:30 h	26.3	7

Table 4Pollinator behaviour recorded on video for *C. virescens* and *B. unilateralis*.^a

Orchid Species	Pollinator	Visits recorded on video	Total flowers visited	Inflorescences visited per population visit	Time spent per inflorescence (s)	Flowers visited per inflorescence	Time spent per flower (s)
<i>C. virescens</i>	<i>B. terrestris</i>	9	22	1.1 ± 0.3 (1–2)	31.2 ± 16.9 (10–60)	2.4 ± 1.4 (1–5)	11.3 ± 5.1 (5–23)
	<i>A. mellifera</i>	8	13	1.1 ± 0.3 (1–2)	53.4 ± 67.7 (10–218)	1.9 ± 0.9 (1–3)	30.0 ± 39.0 (6–157)
<i>B. unilateralis</i>	<i>B. terrestris</i>	7	239	1.8 ± 0.9 (1–4)	65.9 ± 104.7 (6–400)	12.2 ± 16.5 (1–72)	2.9 ± 2.0 (1–14)
	<i>B. ruderatus</i> ^b	3	62	–	–	>13	7.7 ± 5.2 (2–28)

^a Numerical data presented as: Mean ± Standard Deviation (minimum–maximum value).^b It was not possible to obtain information based on inflorescences since the record was fragmented and complete visits were not recorded.**Table 5**Fruiting success and modified Nilsson's male efficiency factor for *C. virescens* and *B. unilateralis*.

Orchid species	Year	Fruiting success ^a	Male efficiency factor ^b
<i>C. virescens</i>	2012	63.65% [64.8% ± 23.1] ^c (883; 77)	0.84 (60.0/71.4; 709; 64)
	2013	67.24% [67.4% ± 22.6] ^c (1050; 97)	0.90 (68.4/75.6; 1025; 94)
<i>B. unilateralis</i>	2012	84.7% [83.2% ± 29.8] (372; 10)	1.59 (72.8/45.8; 603; 16)

^a Presented as: Population's Fruiting Success [mean fruiting success per inflorescence ± standard deviation] (number of flowers used; number of inflorescences used).^b Presented as: Male efficiency factor (% fruiting flowers/% flowers acting as pollen donors; Total number of flowers used to calculate the factor; Total number of inflorescences used).^c Mean fruiting success per inflorescence of *C. virescens* for both years was not statistically different based on a two coiled Mann–Whitney *U*-test ($U = 6518$; $p = 0.746$).

Fig. 1. Floral features of *Chloraea virescens* (Willd.) Lindl. (A) Habit and racemose, multi-flowered inflorescence; (B) Flower with column hidden by petals and labellum bearing green, falcate to lanceolate projections (osmophores); (C) Lateral view of the flower with column exposed showing the position of the anther and stigma relative to the three-lobed labellum; (D) Junction of the column and the ovary showing the two fossae that are characteristic of the genus; (E) Tip of the column with dehiscent anther exposing two friable pollinia adjacent to the triangular stigma with its wide glandular rostellum; (F) Frontal cut of the ovary exposing the two fossae which extend almost half the length of the ovary; nectar is absent. Pollinarium has been removed leaving the anther empty. Scale bars: (A–C) = 1 cm; (D–F) = 1 mm.

(2 × 3 mm) pollinarium with two mealy pollinia, lacking caudicles or viscidium (Fig. 1E and F), and this pollinarium adheres to the pollinator by means of a rostellar secretion (see Section 3.3). Undisturbed and emasculated flowers have a mean longevity of 19 days (Table 1). The flowers emit a subtle, rose-like fragrance that is strongest during the hottest hours of the day.

B. unilateralis has small, greenish-white flowers grouped on a terminal, compact spike which tends to have a second orientation (hence the specific epithet) (Fig. 2A and B). The labellum possesses two thick, median carinae that form a channel leading towards the base of the labellum. The dorsal sepal, along with petals, form a hood that covers the column and most of the labellum, leaving only

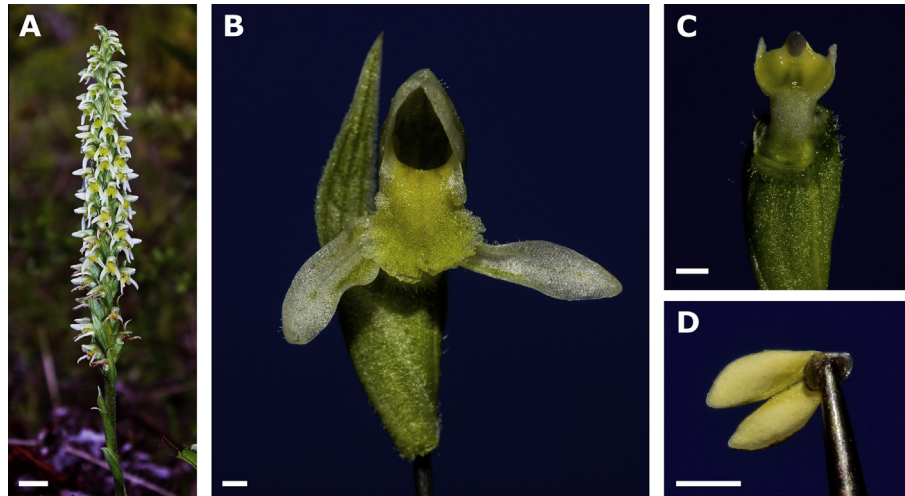


Fig. 2. Floral features of *Brachystele unilateralis* (Poir.) Schltr. (A) Dense, racemose, multi-flowered inflorescence; (B) Frontal view of flower showing dorsal sepal and petals concealing access to the base of the labellum; (C) Short column exposed showing the retinaculum directly above the stigma; (D) Pollinarium comprising a sticky viscidium and two friable, pyriform pollinia. Scale bars: (A) = 1 cm; (B–D) = 1 mm.

the apical reflexed lobe exposed (Fig. 2B). The column is erect, short and robust, the stigmatic surface rounded and subterminal (Fig. 2C). The anther is terminal and produces two pyriform, mealy pollinia which are attached directly to the ventral, discoid viscidium (Fig. 2D). Flowers are protandrous, with a male phase, during which the column extends against the labellum obstructing access to the stigma. This male phase lasts for 10 days, during which flowers act as pollen-donors (see Section 3.3). The female phase begins when the column elevates, exposing the stigmatic surface (Fig. 3). During this last phase, the flowers act as pollen-receivers (see Section 3.3). However, since the surfaces of the viscidia remain sticky, the flowers may also continue to act as pollen-donors. On average, the total flower lifespan is about 16 days (Table 1). Flowers open sequentially and in an acropetal manner and emit a sweet and slightly lemon-like fragrance on warm days. During the first days of anthesis, all open flowers are in the male (pollen-donor) phase. Subsequently, the basal-most flowers enter the female phase (pollen-receiving phase). Thus flowers in both phases may occur simultaneously on the same inflorescence, the younger (more apical) flowers being in the male phase. The flowers offer nectar as a reward and this accumulates directly beneath the column and is possibly secreted by two small, basal, retrorse, hornlike

structures (nectaries, Fig. 3). During the male phase, nectar volume is too small to measure. At the female phase, nectar volume reaches a maximum of 2 μ L with a total sugar concentration exceeding 45% (Table 1).

3.2. Breeding systems

Both species studied were incapable of setting fruit and seed without the intervention of a pollinator, i.e., apomixy and autogamy are absent (Table 2). In *C. virescens* 100% fruit set was obtained following self- and cross-pollination treatments. Fruit set in *B. unilateralis* was not significantly different following self- and cross-pollination treatments. Furthermore, both treatments resulted in a high percentage of fruit set (Table 2). Consequently, both species are completely self-compatible, yet pollinator-dependent.

3.3. Pollinators, behaviour and pollination mechanism

Pollinators recorded for both *C. virescens* and *B. unilateralis* are all members of family Apidae and include: *B. dahlbomii*, *B. ruderatus*, *B. terrestris* and *A. mellifera*. Throughout the whole observation period for *C. virescens* (111 h), only three short visits by *B. dahlbomii* were recorded (Fig. 4A), during which the bee probed up to three flowers per inflorescence and up to two inflorescences from the same population. Indeed, most of the visits recorded were made by *B. terrestris* ($n = 27$), followed by *A. mellifera* ($n = 11$) and *B. ruderatus* ($n = 2$). Visits to populations involved, on average, a visit to a single inflorescence, where 2 or 3 flowers were visited in less than a minute by *Bombus* spp. and the same number in two minutes by *A. mellifera*, following which the bees left the population (Table 4). That pollinators pay particular attention to the *fossae* at the base of the column as they search for nectar is clearly seen (SM 1). Moreover, *A. mellifera* spends some time inspecting the labellar projections (osmophores). In *C. virescens*, the petals and the lateral lobes of the labellum force the pollinator, having alighted on the labellum, to access the flower from the front as it searches for nectar (Fig. 1B, SM 1). The pollinarium becomes attached to the bee when the insect comes into contact with the rostellum. This structure releases a sticky secretion that glues the pollinarium to the dorsum of the pollinator. The pollinarium is then withdrawn from the anther as the bee reverses out of the flower. It is probable that bees become disturbed either by the



Fig. 3. Protandry in flowers of *B. unilateralis*. Sidecuts and frontal views of flowers in different sexual phases. (A) Male phase, where the column extends downwards, thus concealing the stigma and access to the base of the labellum. Note the central channel formed by the two longitudinal ridges; (B) Female phase where column has raised exposing the stigma and allowing easier access to the base of the labellum. Scale bar: 5 mm.

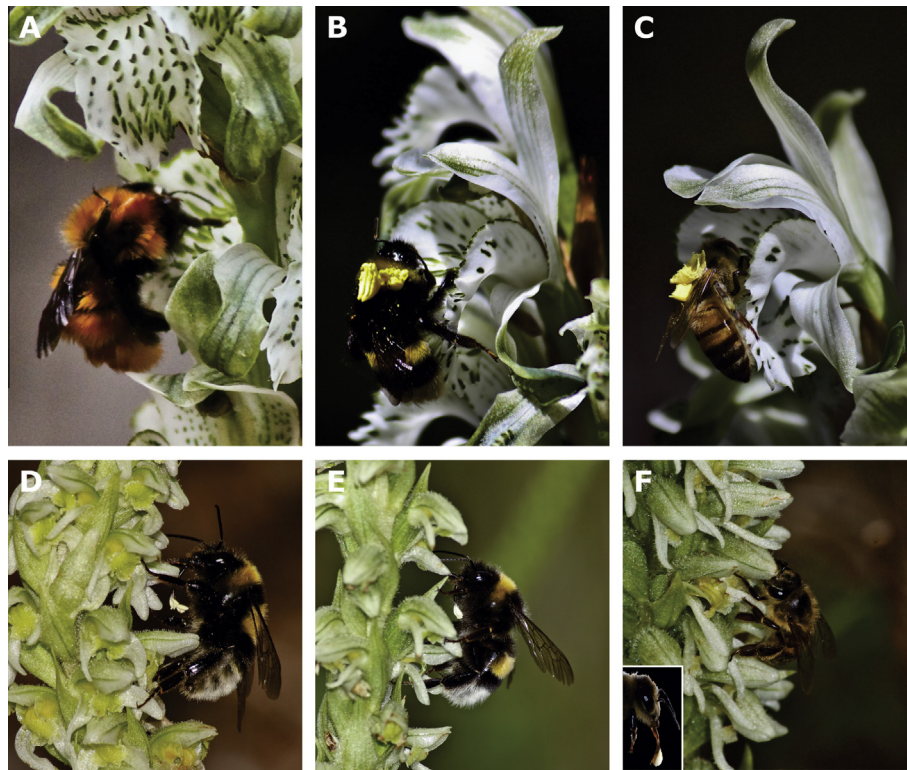


Fig. 4. Pollination of *C. virescens* (A–C) and *B. unilateralis* (D–F). (A) *Bombus dahlbomii* leaving a flower with pollinia remains stuck to the dorsum of its thorax; (B) *Bombus terrestris* worker leaving a flower and carrying two pollinaria, pads of pollen can be seen on the side of the stigma; (C) *Apis mellifera* worker leaving a flower with two pollinaria on its dorsum; (D) Male *Bombus ruderatus* carrying two pollinaria on its proboscis. Note the misshapen bases of the pollinaria, indicating that they have previously been pressed onto a stigma; (E) Male *Bombus terrestris* with a single pollinarium. Note the viscidium attached to its proboscis and the base of the pollinarium also disturbed; (F) *Apis mellifera* worker probing a flower. Inset: *A. mellifera* carrying a pollinarium on its proboscis.

weight of the pollinarium or the rostellar fluid and thus attempt to remove both with their forelegs (SM 1). That said, we have never observed a bee successfully detaching a pollinarium from its body. Pollination occurs when a pollinarium-laden bee enters a flower and the pollinarium comes into contact with the stigmatic surface, resulting in the deposition of pads of pollen on the latter (Fig. 4B and C; SM 1). Often, the bee repeats this action, thereby pollinating many flowers with a single pollinarium, as this structure is large and contains much pollen. Bees can also bear more than one pollinarium at any given time (Fig. 4B and C). The mechanism described above is almost identical for all the *Bombus* species investigated, since they are all similar in size. However, *A. mellifera*, is half the size of *Bombus* species and, on landing on the labellum and entering the flower, its smaller body allows it to move more freely, even laterally, on the labellum and turn itself completely around and exit head foremost, without the need for reversing out of the flower. Unlike *Bombus* spp., pollinaria can adhere to less specific parts of *A. mellifera*, such as the thorax, abdomen or even wings (Fig. 4C; SM 1). Adhesion of a pollinarium to the wing of *A. mellifera* is lethal as it prevents the bee from flying. The smaller size of *A. mellifera* not only permits a greater degree of pollinator movement within the flower, but also reduces the frequency of pollinarium removal. Given the variation in flower size observed in the field, *A. mellifera* has greater opportunity to pollinate those plants with smaller or incompletely opened flowers and those where the distance between the labellum and the apex of the column is relatively short. On this basis, it is reasonable to suppose that *A. mellifera* is a less efficient pollinator of *C. virescens* than are bumblebees. Unidentified colletid bees, and syrphid flies were also observed visiting these flowers and probing the labellar projections, but their bodies were too small to reach and break

the rostellum and to remove the pollinarium. An individual of *Svastrides melanura* (Spinola) (Apidae:Eucerini) bee was once observed visiting a single flower, but was neither able to dislodge pollinaria, nor was it carrying any. However, since this species is similar in size to, or even slightly larger than *A. mellifera*, we speculate that it may also be as an effective pollinator of *C. virescens*.

Recorded pollinators of *B. unilateralis* were mostly *B. terrestris* ($n = 25$), followed by *B. ruderatus* ($n = 15$) and finally by *A. mellifera* ($n = 3$). Just one short visit by *B. dahlbomii* was observed. Pollinators spent, on average, less time probing flowers than in *C. virescens*, although they visited many more flowers per inflorescence (Table 4). It is worth noting that during two years of recording, no visits occurred on the first days of observations, which coincided with the commencement of anthesis, even though pollinators were present and flew close to the flowers. Visits became more frequent once the first visit had been observed. This might be due to the “late” discovery of nectar. In *B. unilateralis*, bees land on the inflorescence and probe flowers with their proboscises (Fig. 4F). If the flower is in male phase (Fig. 3), the column blocks access, forcing the bee to insert its proboscis into the central channel of the labellum so as to reach its basal part. The proboscis thus comes into contact with the ventral viscidium, which almost touches the labellum. The pollinarium is extracted when the bee withdraws its proboscis from the flower (Fig. 4D and E). Attachment of the pollinarium clearly upsets the bee, which then struggles to remove it with its legs before leaving the flower (SM 2). Pollination occurs when a pollinarium-laden bee probes a flower in female phase and the pollinarium makes contact with the stigmatic surface. Pollinaria can also be removed from the flower during this phase and bees can carry more than one pollinarium at a time (Fig. 4D and E). Hesperiid butterflies and ants were observed robbing nectar,

and individuals of *Vespa germanica* (Fabricius) (Vespidae) and *Colletes* sp. (Colletidae) were observed visiting inflorescences without removing pollinaria.

3.4. Natural fruiting success and pollination efficiency

Populations of the orchid species studied demonstrated high fruiting success (Table 5). For *C. virescens*, the mean percentage fruit set per inflorescence was 64.8% and 67.4% for 2012 and 2013, respectively, and these results were not statistically different. In *B. unilateralis*, mean percentage fruit set per inflorescence was 83.2% for 2012. Unfortunately, as it was not possible to record the percentage of fruit set for 2013 (see Section 2.6), it was not possible to compare this parameter statistically for each of the years investigated for this species.

In *C. virescens*, male efficiency factors of 0.84 and 0.90 were recorded for years 2012 and 2013, respectively. A male efficiency factor of 1.59 was recorded for *B. unilateralis* during 2012.

4. Discussion

The general floral structure of *C. virescens* is similar to that of the recently studied *C. membranacea* (Sanguinetti et al., 2012). The falcate to lanceolate labellar green projections react positively to neutral red (Lehnebach and Riveros, 1999), suggesting that they are the osmophores (scent-producing glands) responsible for attracting visitors and produce a slightly rose-like fragrance by day, as does *C. membranacea* (Sanguinetti et al., 2012). It is worth noting, however, that the pair of openings at the base of the column are much more developed in *C. virescens*, whereas they are represented only by two shallow cavities in *C. membranacea*. In neither species do they secrete nectar.

Whereas the general floral structure of *B. unilateralis* is consistent with that of the whole Spiranthinae subtribe (Salazar, 2003), this is the first mention of protandry in the genus. Protandry is well-known and documented for several species of *Spiranthes* L. (Catling, 1982, 1983; Darwin, 1862) and, more recently, has been reported for Neotropical Spiranthinae genera such as *Sauroglossum* Lindl., *Mesadenella* Pabst & Garay and *Skeptrostachys* Garay (Singer, 2002, 2009). In all of these temperate and tropical genera, protandry is known to promote cross-pollination (Catling, 1982, 1983; Darwin, 1862; Singer, 2002, 2009).

Breeding system results (Table 2) are also consistent with previous reports for *Chloraea* species (Humaña et al., 2008; Lehnebach and Riveros, 2003; Sanguinetti et al., 2012) and Spiranthinae (Singer, 2002 and references therein; Singer and Sazima, 2000). Essentially, both orchids studied are unable to set fruit and seed in the absence of pollinators and are completely self-compatible.

Differences in the way that pollinators behave towards these two species (Table 4; SM 1 and 2) can be explained by the fact that *C. virescens* is pollinated by deceit, while *B. unilateralis* offers nectar as a reward. Bees inspect a few flowers of a population of *C. virescens* (*fossae*, osmophores) for nectar before flying away. If the bee withdraws a pollinarium from a previously unvisited flower and subsequently continues visiting flowers from the same inflorescence, then a degree of self-pollination (either as autogamy or geitonogamy) can occur. Conversely, on discovering nectar in *B. unilateralis*, the bee will systematically probe many flowers along the inflorescence (we counted up to 72 in our videos), thus resulting in a much greater rate of self-pollination than occurs in *C. virescens*.

The only native pollinator observed, namely *B. dahlbomii*, accounted for less than 10% of the total visits recorded for both species, the majority of them being performed by the introduced

bees mentioned above, and these also functioned as effective pollinators (Fig 4; SM 1 and 2). It was clear that most of the pollination events observed (e.g. pollinia removal and deposition) were performed by these exotic bees, since these insects conspicuously bore pollinaria and deposited their pollen-content during their visits to the plant populations (SM 1 and 2). Therefore, it is reasonable to state that these aliens are the main pollinators responsible for the high reproductive success recorded at the location under investigation.

The fruiting success of *C. virescens* reported here conforms to that reported for other confirmed, non-autogamous *Chloraea* species. For example, Clayton and Aizen (1996) reported field pollination rates for *C. alpina* of less than 5% and Lehnebach and Riveros (2003) observed a 15.6% fruiting success for *C. lamellata*. Meanwhile, Humaña et al. (2008) demonstrated that fruiting success exceeds 90% in *C. chrysantha* and *C. galeata*, was 33% and 78% for two forms of *C. bletioides*, and approached near 10% for *C. crispa*. Similarly, Sanguinetti et al. (2012) recorded fruiting successes of 42% and 64% for a population of *C. membranacea* during two consecutive years. It was only in *C. chrysantha* and *C. galeata* that natural fruiting was not pollen-limited, i.e. differences between natural fruiting and cross-pollinating by hand were not statistically significant. Unfortunately, no visitors to the flowers were seen and thus, the identity of the pollinator remains unknown (Humaña et al., 2008). The remainder of these orchid species were pollen-limited, that is, there was insufficient pollinator activity for them to achieve their maximum potential fruiting success. Of these plants, species pollinated by scarabids (*C. bletioides*, Humaña et al., 2008), sarcophagid flies (*C. lamellata*, Lehnebach and Riveros, 2003), tabanid flies (*C. bletioides*, Humaña et al., 2008) and halictid bees (*C. lamellata*, Lehnebach and Riveros, 2003; *C. membranacea*, Sanguinetti et al., 2012) displayed greater fruiting success and accordingly, seemed to be less pollen-limited than either *C. crispa* (Humaña et al., 2008) or *C. alpina* (Clayton and Aizen, 1996), species that to-date are known only to be pollinated by *Bombus dahlbomii*. These facts and personal field observations suggest that *Chloraea* species with small flowers that do not open widely are pollinated by a diverse guild of small pollinators such as halictid bees, flies and small to medium scarabs (Sanguinetti, in prep.). By contrast, species with large and widely opened flowers might originally have been pollinated by a restricted guild consisting uniquely of the only native bumblebee, *B. dahlbomii*. No introduced bees were reported at those locations where observations were conducted on *C. crispa* and *C. alpina*, and differences between the abundance of pollinators belonging to these guilds may explain the degree to which pollen-limitation affects fruiting success in non-autogamous species. However, this hypothesis needs further research in order to test the effect of additional factors on the presence and abundance of non-native bees.

No records of fruiting success have previously been published for *Brachystele* species. Therefore, any comparison of reproductive success had to be done on a higher taxonomic level. Based on the compilation of Tremblay et al. (2005), which reports on the natural fruiting success of 10 Spiranthinae species, it would seem that the fruiting success described here for *B. unilateralis* is the highest ever recorded, along with that of *Stenorrhynchos lanceolatus* (Aubl.) Rich. [84.7%, 372 flowers; 10 plants and 83.2%; 438 flowers; 17 plants, respectively], a species exclusively pollinated by hummingbirds (Singer and Sazima, 2000). The average fruiting success for these 10 Spiranthinae species was $17.4\% \pm 30.3$, with many *Spiranthes* spp. showing no fruit formation whatsoever (Catling, 1982). Catling (1983) captured 2 individuals of *A. mellifera* in the USA bearing pollinaria of *Spiranthes vernalis* Engelm. & A. Gray. This, along with a record cited by Catling (1983) based on the work of Coleman (1933), together with our results, are the only examples, of which we are aware, of Apidae bees acting as legitimate orchid

pollinators outside their original range. However, Catling (1982) reported a null fruiting success for *S. vernalis*, which may indicate that *A. mellifera* is not as efficient in pollinating this orchid species.

Tremblay et al. (2005) compiled fruit production for 216 non-autogamous orchids, of which 124 species belong to temperate climates, 71 are pollinated by deceit and the remaining 53 offer some kind of reward. Generally, the reproductive success of temperate climate orchids is double that of tropical species (34.6% vs. 17.0%, respectively), whereas that of deceit-pollinated species is only half that of species that offer rewards (20.7% vs. 37.1%, respectively). Based on this compilation, our results place *C. virescens* in the top 5% of deceit-pollinated, temperate climate orchids and position *B. unilateralis* in the top 10% of rewarding, temperate climate species (Table 6).

We believe that the high reproductive success of these species is directly linked to the high incidence of non-native bees observed in the field, as compared with the almost complete absence of *B. dahlbomii*, and their status as generalist pollinators (Goulson, 2003; Traveset and Richardson, 2011). As a result, the populations are visited many times, resulting in a high pollination rate. Both characteristics, namely, their great abundance and generalist status, provide them with the ability to exert disproportional effects on plant-pollinator interactions relative to the small proportion of pollinator diversity that they represent, which is why they are considered “super-generalists” (Morales et al., 2009). As an analogous example, recent studies of the temperate Patagonic forests (Aizen et al., 2011; Madjidian et al., 2008) have shown that the introduced *B. ruderatus* is actually the most effective pollinator of native *Alstroemeria aurea* Graham (Alstroemeriaceae). Despite being less efficient than their main native pollinator, *B. dahlbomii* (Aizen, 2001), this species of bee is superior in effectiveness – measured as the reproductive success of *A. aurea* – by achieving much higher visiting frequency rates (Madjidian et al., 2008). Moreover, these bees are less selective when foraging for nectar, while *B. dahlbomii* is more selective and consequently more efficient at nectar foraging (Aizen et al., 2011). The “hyperactive” behaviour of *B. ruderatus*, in terms of its greater visiting frequency rates and lower preference for particular nectar, is shared with *B. terrestris* and *A. mellifera* (Aizen et al., 2008; Goulson, 2003; Ings et al., 2006; Morales, 2007). Consequently, the investigated orchids may benefit in a context of resource scarcity (see below).

In addition, there are other factors that appear to contribute to the reproductive success of these orchids. Since insect-mediated self-pollination (autogamy and geitonogamy) was observed in both species, especially in *B. unilateralis*, self-compatibility guarantees fruiting at every pollination event, regardless of the origin of the pollinarium. By contrast, if these species were self-incompatible, many ovaries would abort when pollinated with pollinaria from the same plant, and thus, fruit set would be lower (Larson and Barrett, 2000). These orchids flower in late summer when there is a paucity of other plants offering flowers and nectar (Vázquez and Simberloff, 2004). Indeed, no other species in bloom were seen at the study site, with the exception of a few and distant individuals of *Gevuina avellana* Molina (Proteaceae), *A. aurea* and *Mutisia decurrens* Cav. (Asteraceae). The reproductive success of both orchids would be different if flowering occurred in spring, when more abundant floral resources are available. Another factor, especially for *C. virescens*, is the floral longevity of both emasculated and intact flowers. A 19 day period of fresh flowers increases the opportunity for visits and pollination events (Primack, 1985). This floral longevity is a trait shared with many European deceptive orchids which maximize pollination success in this way (Neiland and Wilcock, 1995).

Primack and Stacy (1998) and Snow and Whigham (1989) reported that repeated high fruiting episodes in the orchids *Cypripedium acaule* Aiton (Cypripedioideae) and *Tipularia discolor*

Table 6

Fruiting success per inflorescence for rewarding and deceit pollinated orchid species of temperate climates based on Tremblay et al. (2005), together with *C. virescens* and *B. unilateralis*.

	Temperate orchids			<i>C. virescens</i>	<i>B. unilateralis</i>
	n	Mean	SD		
Deceptive	71	29.3%	20.8	66.1%	–
Reward	52	41.5%	29.9	–	83.2%

(Pursh) Nutt. (Epidendroideae), respectively, reduced subsequent vegetative growth and hence the probability of further flowering. Conversely, Calvo (1990) did not find negative effects of reproduction in the following season after fructification on *Cyclopogon cranichoides* (Griseb.) Schltr. (Orchidoideae), however this was not assessed for consecutive years. The consequences of such high reproductive success in *C. virescens* and *B. unilateralis* (Orchidoideae) individuals remain to be tested on a long-term basis within experiments ranging from 4 to 7 years as suggested in Primack and Stacy (1998).

Nowadays it is almost impossible to estimate the fruiting success of *C. virescens* and *B. unilateralis* in the absence of the introduced pollinators, on account of their expansion across the range of these orchids and the lack of reports on the pollination and fruiting of these orchids that pre-date the introduction of the bees. Nevertheless we estimate that given the actual context of the decline of *B. dahlbomii*, the fruiting success of the orchids would be significantly lower had these introduced bees been absent from the studied populations. Moreover, in the case of *C. virescens*, as in *C. crispera* and *C. alpina*, which are both pollinated solely by the native bumblebee (Clayton and Aizen, 1996; Humaña et al., 2008), low reproductive success can be sustained. Visits by *B. dahlbomii* were very few during our observations, probably due to the low abundance of this insect in the field (Montalva et al., 2011; Morales et al., 2013). Therefore, in its declining state, this native bee species is unlikely to provide a pollination service as effective as that promoted by introduced bees.

Calculated male efficiency factors (Table 5) are consistent with the different ways pollinators behave towards the studied orchid species (see Section 3.3); a Nilsson's factor of less than one indicates that there is a certain degree of pollen loss in the system of *C. virescens* (Singer and Cocucci, 1997). *B. unilateralis*, however, has a Nilsson's factor of greater than one indicating that more than one flower can successfully set fruit per pollinarium removed, which, in turn suggests high male efficiency, and this can be explained in terms of pollinator behaviour. Although *B. unilateralis* is protandrous, a structural mechanism favouring cross-pollination (Johnson and Edwards, 2000; Singer, 2002), here, protandry does not appear to be very efficient, since pollinators rarely behave the way one would expect for this system to work (i.e. probing inflorescences from base to apex and then departing (see Fig. 5 in Catling, 1983)). Thus, a great proportion of the fruiting of *B. unilateralis* must be due to self-pollination, while the converse occurs in *C. virescens*. Therefore, the results available to date support the hypothesis that when pollinators are common, the absence of nectar serves to reduce the occurrence of pollinator-mediated geitonogamy, thereby avoiding the negative consequences of self-pollination (Jersáková et al., 2006, and references therein).

Ironically, the introduction of *B. ruderatus* and *B. terrestris* which now seems to be a blessing for these orchids might have been a curse for them since there is strong evidence (Arbetman et al., 2012; Madjidian et al., 2008; Montalva et al., 2011; Morales, 2007; Morales et al., 2013) that these exotic bumblebees are involved in the abrupt retraction and disappearance of *B. dahlbomii*. In a plausible scenario of *B. dahlbomii* extinction these exotic species would functionally substitute and completely overcome

the pollination service of *B. dahlbomii*. Similar cases are well documented, for example, with non-native avifauna serving as pollinators and seed dispersers for Hawaiian native plant species whose flowers were once pollinated and whose seed were once dispersed by now-extinct native birds (Cox, 1983; Foster and Robinson, 2007); Fox et al. (2013) recently reported the case of an orchid recruiting a non-native pollinator introduced for weed control, and it is this species that is currently responsible for most of the reproductive success of the orchid owing to its greater abundance as compared with the four native pollinators. Similarly, Downing and Liu (2012) reported that the recently introduced *Centris nitida* Smith (Apidae) acts as a legitimate and better pollinator of *Byrsonima lucida* (Mill.) DC. (Malpighiaceae) than is *C. errans* Fox, its threatened and only native pollinator. Even invasive black rats (*Rattus rattus* L.) have been shown to contribute to the success of some of New Zealand's endemic trees, despite the fact that they are also the reason for the loss of native pollinators (Pattimore and Wilcove, 2012). All in all, this novel interaction might promise a better outlook for these and other bumblebee-pollinated, regional orchid species, owing to the ability of invasive species to tolerate and adapt to a broad range of biotic and abiotic conditions (Schlaepfer et al., 2011, and references therein) together with a speculated increased resistance to disturbance (Traveset and Richardson, 2011, and references therein).

Although it is quite clear that these introduced key pollinators enable novel interactions that currently benefit the studied orchids, it must also be recognized that they are also probably responsible for greatly rearranging the pollination web that they have invaded (Aizen et al., 2008; Traveset and Richardson, 2011; Traveset et al., 2013). Moreover, it is possible that these interactions are subject to novel ecological and evolutionary dynamics (e.g., the lethal effect of the pollinarium of *C. virescens* on *A. mellifera*) and that their effects are transitory (Vitule et al., 2012). A longer-term assessment on a greater scale is necessary in order to determine whether the effects of these invaders last or whether the reproductive success of the orchids fluctuates or diminishes (Strayer et al., 2006). Besides this, efforts to conserve *B. dahlbomii* should be encouraged and supported in order to avoid it becoming extinct.

5. Conclusion

In this study, we show that pollination in *C. virescens* and *B. unilateralis*, which may have been primarily performed by *B. dahlbomii*, has been taken over by alien agents that are functionally similar to this species, such as *B. ruderatus*, *B. terrestris* and *A. mellifera* (Fig. 4; SM 1 and 2). Moreover, the reproductive success of this novel interaction is surprisingly high compared with previous studies on orchids (Table 6).

To our knowledge, after Coleman (1933), Catling (1983) and Fox et al. (2013), this is the fourth report on alien agents successfully acting as orchid pollinators, but the first involving bumblebees (*Bombus* spp.). In addition, this is the first report of pollination in a species of *Brachystele*. Increased diversity and abundance of generalist pollinators morphologically suited for the pollination of these orchids (e.g. the large body size of pollinators of *C. virescens* and the long and stiff proboscis of pollinators of *B. unilateralis*) have resulted in the current excellent fruiting performance described here for both species.

Finally, there is a caveat – namely, that any eventual conservation plan that involves the population management of these introduced bumblebees (since the reproductive success of the particular orchids discussed here largely depends on them) should be undertaken with care. This, however, should by no means be seen as a *carte blanche* to introduce exotic bumblebees wherever it is thought that they could improve the fruiting success of bumble-

bee-pollinated orchids, since this too could precipitate detrimental consequences at the community level (Morales et al., 2013; Vitule et al., 2012).

Acknowledgements

We thank The Rufford Foundation for funding, National Parks Administration of Argentina (APN) for research and collecting permit N° 1183, the Lago Puelo National Park staff (M. Ocampo & N. Zermatten) for logistical support and Claudia Riccardi and family for accommodation and general support in Lago Puelo. We also thank Sara Maldonado, and three anonymous reviewers who helped us improve a first version of this manuscript; Kevin L. Davies (Cardiff University) helped to improve the English text; Mariela Lacoretz provided assistance in statistical analyses. AS acknowledges Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) for his scholarship and thanks Ideawild for donating field equipment.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocon.2014.04.011>. These data include Google maps of the most important areas described in this article. Both videos on pollination of *C. virescens* (SM1) and *B. unilateralis* (SM2) can also be found respectively at <http://youtu.be/ny3QLgfC3rs> and <http://youtu.be/sONmYFPkco4>.

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