

## Caught in the act: pollination of sexually deceptive trap-flowers by fungus gnats in *Pterostylis* (Orchidaceae)

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• **Background and Aims** *Pterostylis* is an Australasian terrestrial orchid genus of more than 400 species, most of which use a motile, touch-sensitive labellum to trap dipteran pollinators. Despite studies dating back to 1872, the mechanism of pollinator attraction has remained elusive. This study tested whether the fungus gnat-pollinated *Pterostylis sanguinea* secures pollination by sexual deception.

• **Methods** The literature was used to establish criteria for confirming sexual deception as a pollination strategy. Observations and video recordings allowed quantification of each step of the pollination process. Each floral visitor was sexed and DNA barcoding was used to evaluate the degree of pollinator specificity. Following observations that attraction to the flowers is by chemical cues, experimental dissection of flowers was used to determine the source of the sexual attractant and the effect of labellum orientation on sexual attraction. Fruit set was quantified for 19 populations to test for a relationship with plant density and population size.

• **Key Results** A single species of male gnat (Mycetophilidae) visited and pollinated the rewardless flowers. The gnats often showed probing copulatory behaviour on the labellum, leading to its triggering and the temporary entrapment of the gnat in the flower. Pollen deposition and removal occurred as the gnat escaped from the flower via the reproductive structures. The labellum was the sole source of the chemical attractant. Gnats always alighted on the labellum facing upwards, but when it was rotated 180° they attempted copulation less frequently. Pollination rate showed no relationship with orchid population size or plant density.

• **Conclusions** This study confirms for the first time that highly specific pollination by fungus gnats is achieved by sexual deception in *Pterostylis*. It is predicted that sexual deception will be widespread in the genus, although the diversity of floral forms suggests that other mechanisms may also operate.

**Key words:** Orchid, pollination, sexual deception, fungus gnat, *Pterostylis*, semiochemicals, specialization, Mycetophilidae.

### INTRODUCTION

Sexual deception is a pollination strategy in which rewardless flowers secure pollination by the sexual attraction of male insects by chemical and/or physical mimicry of a female, with pollination often achieved during attempted courtship or copulatory behaviour (Peakall, 1990; Schiestl, 2005; Gaskett, 2011). While primarily known from the Orchidaceae (Schiestl, 2005; Gaskett, 2011), sexual deception has recently been discovered cases in the Asteraceae (Ellis and Johnson, 2010) and the Iridaceae (Vereecken *et al.*, 2012), suggesting that the phenomenon may be much more taxonomically widespread than currently appreciated. Within the Orchidaceae, work in comparatively poorly studied tropical floras has revealed several new cases of sexual deception, greatly increasing the range of orchid tribes and pollinator groups involved (Singer, 2002; Singer *et al.*, 2004; Blanco and Barboza, 2005; Ciotek *et al.*, 2006). Taken together, these discoveries suggest that the evolution of sexual deception may be much more frequent than presently documented.

As a consequence of mimicking the specific sexual signals of female insects, pollination by sexual deception is a highly specialized strategy, with most species reliant on the chemical attraction of just a single pollinator species (Paulus and Gack, 1990; Phillips *et al.*, 2009; Peakall *et al.*, 2010; Phillips, 2010). However, as sexually deceptive systems are studied in increasing depth, a diversity of floral adaptations is being revealed. For example, the chemical attractants involved in the long-range attraction of pollinators encompass a range of chemical classes, such as chiloglottones (2,5-dialkylcyclohexane-1,3-diones), pyrazines, unsaturated hydrocarbons and oxygenated acids (Schiestl *et al.*, 1999, 2003; Ayasse *et al.*, 2003; Franke *et al.*, 2009; Bohman *et al.*, 2012a, b). The importance and role of floral colour likely vary between systems, but at short range it may enhance attraction by direct mimicry (Gaskett and Herberstein, 2010), maximizing colour contrast to aid detectability (Streinzer *et al.*, 2009) or exploiting sensory bias (Gaskett, 2011). Using a case of pollinator sharing between the unrelated sexually deceptive orchids *Drakaea livida* and *Caladenia*

*pectinata*, Phillips et al. (2013) showed that floral traits that affect pollinator behaviour have direct consequences for plant fecundity. In *D. livida*, which releases its sexual attractant from the labellum, there is higher pollination success than in *C. pectinata*, which produces its attractant from sepals distal from the reproductive parts of the flower (Phillips et al., 2013). Consequently, understanding the range of floral adaptations to pollinator behaviour will prove important for determining the traits underpinning the evolution and function of sexually deceptive systems.

While most sexual deceptive pollination systems involve Hymenoptera (Schiestl, 2005), there are many orchid genera pollinated by Diptera that may harbour such systems. For example, in the Pleurothallidinae, which contains over 4100 species, almost all records of pollination concern Diptera, but the mechanism of pollinator attraction has been resolved in only a handful of species (Pridgeon, 2005; but see Borba and Semir, 2001; Barbosa et al., 2009; Melo et al., 2011). Until now, the only well documented cases of pollination by sexual deception of Diptera in the Orchidaceae are in the large neotropical orchid genus *Lepanthes* (>700 species; Blanco and Barboza, 2005). Observations of the pollinator behaviour in *Lepanthes* revealed that the courtship and copulatory behaviour of sciarid fungus gnats explained floral adaptations not seen in other sexually deceptive systems. For example, the presentation of the flowers against the surface of the leaf matches the calling position of the female gnat (Blanco and Barboza, 2005). Consequently, the discovery of sexual deception of Diptera by other orchids is expected to yield important insights beyond those achieved for species pollinated by Hymenoptera.

The diverse Australasian orchid genus *Pterostylis* (>400 species; Jones, 2006; Brown et al., 2008) has long been known to be pollinated by small Diptera (Sargent, 1909), with most recorded floral visitors being members of the Mycetophilidae (Hyett, 1960; Bernhardt, 1995; Brown et al., 1997; Gaskett, 2011; but see Coleman, 1934). However, despite a history of study dating back to the 19th century (Cheeseman, 1872), the mechanism of pollinator attraction has not been resolved for a single species (Adams and Lawson, 1993; Gaskett, 2011). All species investigated are recorded as being nectarless and use a touch-sensitive, motile labellum to trap pollinating insects within a galeate fusion of the lateral and dorsal sepals (Sargent, 1909; Erickson, 1951; Jones, 2006). While all species are dull green, brown or red-brown, there is considerable variation in floral morphology, leading to sexual deception and shelter site both being suggested as potential pollination strategies (Adams and Lawson, 1993; Bernhardt, 1995). In particular, in some species complexes, the insectiform labella and anecdotal accounts of pollinator behaviour (Jones, 2003; Gaskett, 2011) are suggestive of the strategy of sexual deception.

Our discovery of male fungus gnats as the pollinators of *Pterostylis sanguinea* provided an opportunity to objectively assess whether or not pollination by sexual deception might be operating in *Pterostylis*. Because claims of sexual deception in *Pterostylis* are either speculative or based only on anecdotal observations, we first established a set of criteria that characterizes other sexually deceptive systems. We conducted observations and experiments to assess the case for sexual deception in *P. sanguinea* against these objective criteria. The sexual behaviour of the pollinator with the flower was quantified, and we used

experiments to investigate the role of floral odour and morphology in sexual attraction. Using DNA barcoding of insects attracted to the orchid, we tested for the high pollinator specificity evident in other sexually deceptive systems. We also evaluated the relationships of fruit set with population size and density to understand the ecological consequences of pollination by sexual deception of fungus gnats.

## MATERIALS AND METHODS

*Pterostylis sanguinea* is a common and widespread species ranging across south-western Australia, southern South Australia, Victoria and Tasmania (Jones, 2006; Hoffman and Brown, 2011). Flowering occurs in winter, with peak flowering in June and July (Hoffman and Brown, 2011). Two to five flowers are typically produced per stem, but large individuals can produce 12 or more flowers with up to four flowers open at any one time (Fig. 1; Hoffman and Brown, 2011). Flower colours range between red-brown and green, with various colour forms often co-occurring within a population. *Pterostylis sanguinea* is self-compatible but reliant on a pollen vector for pollination (Retter, 2009). Thus far, the pollinator of *P. sanguinea* has not been recorded, although an unidentified species of fungus gnat (Mycetophilidae) has been found dead in some flowers (Retter, 2009).



FIG. 1. *Pterostylis sanguinea*, illustrating flowers and growth habit. In the study region, larger individuals regularly grow to 40 cm. Photograph by R. D. Phillips.





FIG. 2. Demonstration of the hinge mechanism and internal structure of the galea of *Pterostylis sanguinea*. (A) Flower with the hinge ready to trap a pollinator. (B) The labellum has triggered and moved into the 'up' position and the column wing has been removed to show the passage through which the gnat must move to exit the flower. The flower is approximately 10 mm across the galea. Photographs by K. W. Dixon.

Observations of the trap mechanism and movement of the pollinator through the flower have been published for the closely related *Pterostylis vittata* (Sargent, 1909). However, based on the region where Sargent made his observations, it is likely that these plants were actually *P. sanguinea*. For pollination to occur, the pollinator must make contact with the labellum, which then swings upwards, trapping the gnat within the galea (Sargent, 1909) (Fig. 2). The labellum remains in this position, keeping the pollinator trapped within the flower, where it flies upwards, keeping it in regular contact with the column (Sargent, 1909). After several minutes the pollinator crawls through the passage formed by the column wings (lined with angled bristles to ensure one-way movement of the insect), bringing it into contact with the lightly hinged anther, which then deposits the pollinia as it leaves the flower (Sargent, 1909) (Fig. 2). This one-way passage of the pollinator effectively prevents self-pollination. Sargent (1909) estimated that the labellum takes approximately 2 h to reset, but noted that the time was delayed by cool conditions.

#### Confirmation and description of pollination by sexual deception

**Criteria for confirming sexual deception.** We define pollination by sexual deception as a strategy in which plants lure pollinators with signals that are sexually attractive to the pollinator. While there is considerable diversity among sexually deceptive systems, meeting one or more of the criteria listed below provides confirmation of sexual deception (Table 1). It should be noted that the level of sexual response can vary markedly between individual visitors, meaning that in many cases only a portion of the insects arriving at a flower will show the full potential repertoire of sexual behaviour at the flower (e.g. approach, alight and attempted copulation; Peakall, 1990; Phillips *et al.*, 2013). Further, the level of the sexual response can also vary with the time of day and stage in the season (Paulus and Gack, 1990).

(1) *Pre-mating behaviour.* Any behaviour associated with courtship (e.g. wing fanning of gnats towards *Lepanthes*; Blanco and Barboza, 2005) or the initiation of mating

behaviour towards the flower (e.g. male thynnine wasps attempting to grasp a *Drakaea* labellum while in flight; Peakall, 1990). The identification of pre-mating behaviour requires an understanding of the sexual behaviour of mating pairs of the pollinator.

- (2) *Attempted copulation.* An attempt to initiate copulation with the flower (e.g. Coleman, 1928; Kullenberg, 1961; Peakall, 1989; Paulus and Gack, 1990; Schiestl *et al.*, 1999; Blanco and Barboza, 2005; Phillips *et al.*, 2013).
- (3) *Ejaculation.* Sperm release during attempted copulation with the flower. Ejaculation is known for pollinators of two sexually deceptive orchid genera (Blanco and Barboza, 2005; Gaskett *et al.*, 2008), but appears unlikely to occur in most others.
- (4) *Chemical mimicry of sex pheromones.* Flowers produce semiochemicals that mimic the chemical composition of the sex pheromone of the pollinator (e.g. Schiestl *et al.*, 1999, 2003; Stokl *et al.*, 2007; Ayasse *et al.*, 2011). Bioassays with synthetic semiochemicals are required to confirm that the specific compounds (or specific blends) elicit sexual attraction and sexual behaviour (e.g. Peakall *et al.*, 2010). Sexual deception may still be involved when a pollinator exhibits a flight pattern indicative of tracking an odour plume, but fails to show any subsequent sexual behaviour at the flower. This distinction may be particularly important in cases where entrapment may prevent the full repertoire of sexual behaviour. Here, sexual attraction could be tested by presenting the scent-producing parts in isolation from the remainder of the flower or by adding chemical extracts from the flower to a dummy female.

In the absence of observations that meet the above criteria, other lines of evidence may be indicative of pollination by sexual deception. However, these traits alone are insufficient to confirm sexual deception (Table 1).

(1) *Males only.* All known cases of sexual deception involve the attraction of male pollinators (Gaskett, 2011), but female-only attraction remains a possibility. It is worth

TABLE 1. Evidence required for confirmation that pollination occurs via sexual deception

Species	Pollinator	Characteristics of sexual deception											Reference	
		MALE	POL (N)	REWARD	INFORM	Chemical attractant			Confirmation of sexual deception			Pheromone		
						RA	OP	EX	PRE-MATE	COPULA	EJAC			
<b>Orchidaceae</b>														
<i>Caladenia pectinata</i>	Thynnine wasp	Yes	1	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Not likely	Unknown	Phillips <i>et al.</i> (2013)
<i>Calochilus holtzei</i>	Scoliid wasp	Yes	1?	No	Yes	Yes	X	X	Yes	Yes	Yes	Not likely	Unknown	Jones and Gray (1974)
<i>Chiloglottis trapeziformis</i>	Thynnine wasps	Yes	2	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Not likely	Chiloglottones	Schiestl <i>et al.</i> (2003)
<i>Cryptostylis erecta</i>	Ichneumonid wasp	Yes	1	No	Yes	Yes	Yes	Yes	?	Yes	Yes	Yes	Unknown	Gaskett <i>et al.</i> (2008)
<i>Disa atricapilla</i>	Sphecid wasp	Yes	1	No	No	X	Yes	X	?	No	No	Unknown	Steiner <i>et al.</i> (1994)	
<i>Drakaea glyptodon</i>	Thynnine wasp	Yes	1	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Not likely	Pyrazines	Peakall (1990), Bohman <i>et al.</i> (2012a)
<i>Geoblasta penicillata</i>	Scoliid wasp	Yes	1	No	Yes	X	Yes	X	?	Yes	Yes	Not likely	Unknown	Ciotek <i>et al.</i> (2006)
<i>Lepanthes glicensteinii</i>	Sciarid gnat	Yes	1	No	Yes	X	Yes	X	Yes	Yes	Yes	Yes?	Unknown	Blanco and Barboza (2005)
<i>Leporella fimbriata</i>	Formicid ant	Yes	1	No	Yes	No	Yes	Yes	?	Yes	Yes	Not likely	Unknown	Peakall (1989)
<i>Mormolyca ringens</i>	Apid bees	Yes	2	No	Yes	X	X	X	?	Yes	Yes	Not likely	Alkenes and alkanes?	Singer <i>et al.</i> (2004); Flach <i>et al.</i> (2006)
<i>Ophrys sphegodes</i>	Andrenid bee	Yes	5?	No	Yes	Yes	Yes	Yes	?	Yes	Yes	Not likely	Alkenes and alkanes	Schiestl <i>et al.</i> (1999), Gaskett (2011)
<i>Orchis galilaea</i>	Halictid bee	Yes	1	No	No	X	Yes	X	No	No	No	Unknown	Unknown	Bino <i>et al.</i> (1982)
<i>Paracaleana nigrata</i>	Thynnine wasp	Yes	X	No	Yes	Yes	Yes	X	X	X	X	Unknown	Unknown	Hopper and Brown (2006)
<i>Pterostylis sanguinea</i>	Mycetophilid gnat	Yes	1	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Not likely	Unknown	Present study
<i>Serapias lingua</i>	Apid bee	Yes	1?	No	Yes	X	X	X	?	Yes	Yes	Not likely	Unknown	Vereecken <i>et al.</i> (2012)
<i>Trigonidium obtusum</i>	Meliponine bee	Yes	1	No	No	X	No?	X	?	Yes	Yes	Not likely	Unknown	Singer (2002)
<i>Spiculaea ciliata</i>	Thynnine wasp	Yes	1	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Not likely	Unknown	Alcock (2000)
<b>Asteraceae</b>														
<i>Gorteria diffusa</i>	Bombyliid fly	No	1	Yes	Yes	No	No	X	Yes	Yes	Yes	No	No pheromone?	De Jager and Ellis (2012)
<b>Iridaceae</b>														
<i>Iris paradoxa</i>	Apid bee	Yes	1?	X	Yes	X	X	X	Yes	Yes	Yes	Not likely	Unknown	Vereecken <i>et al.</i> (2012)

Examples from the literature are used to show the characteristics of sexual deception and identify gaps in the literature: X, this element has not been tested or quantified; MALE, only males are pollinators; POL (N), number of pollinator species. REWARD, a reward is provided to the pollinator; INFORM, insectiform flower.

A chemical attractant can be confirmed with three lines of evidence: RA, rapid attraction of pollinators; OP, the pollinator behaves as if it is tracking an odour plume; EX, experiments such as covering of flowers. PRE-MATE, pre-mating behaviour such as courtship signals or grasping prior to copulation; COPULA, attempts copulation with the flower; EJAC, ejaculates on the flower.

Note that (1) not all pollinators responding to a given species show the full repertoire of behaviours, and (2) data in this table only relate to pollinators attracted by sexual deception.

noting that for some insects, such as thynnine wasps, in which the females are wingless, males are the only sex available as potential pollinators (Peakall, 1990). Therefore, when observing pollination by males only, the biology of the insect species needs to be considered before interpreting this as evidence for sexual deception.

- (2) *Food reward absent.* There are no confirmed cases of sexually deceptive orchids rewarding pollinators with nectar. However, in *Gorteria diffusa* (Asteraceae), male and female pollinators take nectar and pollen from flowers, while males are also sexually attracted to the dark spots present in some morphotypes (De Jager and Ellis, 2012).
- (3) *Highly specific.* In most species, only one or two pollinator species are attracted via sexual deception (see review in Gaskett, 2011). However, in some sexually deceptive orchids, food-foraging insects can potentially act as pollinators (Steiner et al., 1994).
- (4) *Chemical attractant.* All known sexually deceptive orchids attract their pollinators via floral odour. By contrast, in *G. diffusa* attraction of sexually deceived pollinators is by visual cues (De Jager and Ellis, 2012). Chemical attraction can be confirmed by attracting pollinators to covered flowers (Kullenberg, 1961), an approach flight associated with insects following an odour plume (Stoutamire, 1983) or the rapid attraction of males to floral odours released from artificially presented flowers (Peakall, 1990).
- (5) *Insectiform floral structure.* Most species of sexually deceptive orchids have an insectiform floral structure (e.g. Coleman, 1928; Kullenberg, 1961; Stoutamire, 1974; Paulus and Gack, 1990), though there are exceptions (e.g. Phillips et al., 2009). We consider insectiform orchid flowers to have some or all of the following traits: dull coloured, inconspicuous flowers; reduced petals and sepals; a large labellum relative to the remaining petals and sepals; the presence of hairs and/or a pronounced texture. In the case of *G. diffusa*, dark floral spots on the otherwise brightly coloured ray florets are associated with sexual behaviour (De Jager and Ellis, 2012).

*Description of behaviour of the pollinator on the flower.* Observations of pollinator behaviour were made in Kings Park, Western Australia, with additional observations at nearby Star Swamp Reserve (Table 2). At both sites, *P. sanguinea* was present. Flower-visiting gnats were most active between 1000 and 1600 h, though flight times were delayed on cold mornings (e.g. <5 °C). All observations were made between 1000 and 1600 h across 7 days between 21 June and 11 July 2013 (487 min of observation in total). During the observation periods temperature ranged between 17.5 and 22 °C, as measured with a Tinytag data logger (Gemini Data Loggers) suspended 30 cm above the ground (approximating flower height).

We conducted observations of pollinator behaviour based on the baiting method of Stoutamire (1974) and Peakall (1990), in which picked flowers are used to attract pollinators. As observed in sexually deceptive orchids pollinated by thynnine wasps, re-locating bait flowers to a new location initiates a renewed response. Picked flowers were maintained in vials of water at all times and stored in a refrigerator at 4 °C between experiments. Three flowering stems were used simultaneously as an attractant, with one to three flowers open per stem. We baited at random positions within the population of fungus gnats, with consecutive positions greater than 3 m apart. Baiting was undertaken for 5-min periods in each position. If no gnats were attracted during this period, orchids were moved to another position. If gnats were attracted, we continued to make observations until visitation ceased. A total of 32 observation periods were conducted.

Pollinator behaviour at the flower was recorded for subsequent detailed evaluation with a Sony Digital HD Video Camera Recorder. Since no courting or mating pairs were observed during the study, the curling of the abdomen below the thorax was taken as evidence of courtship behaviour [as illustrated in Blanco and Barboza (2005) for sciarid gnats], while vigorous probing with the tip of the abdomen was interpreted as attempted copulatory behaviour. The position of the abdomen during copulatory probing was quantified for 29 separate visits. Probes were classed as being made at the labellum apex, along the lower

TABLE 2. Study populations used to survey for pollinators of *Pterostylis sanguinea*

Site	Latitude/longitude	Flowers dissected (late June)	Flowers dissected (mid-July)	Minutes of baiting	Gnats with pollen
Alfreton Reserve	31-82691 °S, 115-78108 °E	44	Not surveyed	<b>60 (1)</b>	0
Breckler Park	31-89863 °S, 115-86318 °E	55	35	Not surveyed	0
Kings Park, population 1	31-95623 °S, 115-83578 °E	30	Not surveyed	60	0
Kings Park, population 2	31-95469 °S, 115-83208 °E	<b>25 (1)</b>	21	60	0
Kings Park, population 3	31-96215 °S, 115-82569 °E	Not surveyed	10	Not surveyed	0
Kings Park, population 4	31-95727 °S, 115-84156 °E	Not surveyed	Not surveyed	<b>307 (134)</b>	6
Paloma Park	31-86520 °S, 115-75229 °E	20	32	Not surveyed	0
Pinnaroo Valley	31-81411 °S, 115-77867 °E	<b>20 (1)</b>	20	60	1
Shepherds Bush Reserve	31-81004 °S, 115-79347 °E	33	20	60	0
Shenton Park	31-8004 °S, 115-79347 °E	<b>32 (2)</b>	25	60	1
Star Swamp Reserve, population 1	31-85824 °S, 115-75948 °E	<b>33 (2)</b>	<b>30 (1)</b>	<b>120 (25)</b>	2
Star Swamp Reserve, population 2	31-85464 °S, 115-76125 °E	11	8	Not surveyed	0
Trigg Reserve	31-87030 °S, 115-76025 °E	31	35	60	0
Total		334 (6)	236 (1)	487 (160)	10

Numbers in parentheses are the number of gnats detected.  
Bold indicates the surveys at which gnats were detected.



section of the surface of the labellum, towards the upper section of the labellum or immediately below the callus (Fig. 3).

During field observations, pollinator behaviour was quantified across a hierarchy of responses following Peakall (1990) and Phillips *et al.* (2013). For each floral visitor (Fig. 4) we recorded the presence of pollen on arrival, whether or not the pollinator touched the labellum, the duration of any attempted copulation and the incidence of labellum triggering. If the labellum was triggered, we recorded the duration of pollinator entrapment and whether or not pollination was achieved. For each visitor, the time taken to arrive at an artificially presented bait flower and the total time spent on the flower were also recorded.

Due to the difficulty of observing such small insects in the wild (body length of approximately 3.5 mm), it was only possible to observe their approach to the flower when the sun was low in the sky and so illuminating their wings in flight. Therefore, we could only make general observations of how they approached the flower.

*How long does the labellum take to reset?* On 12 July we conducted an experiment in Kings Park bushland to quantify how long it takes the labellum to reset after being triggered. The experiment was conducted between 1430 and 1630 h, coinciding with the period of peak gnat activity. Temperature ranged between 15.7 and 19.1 °C in sunny weather, following rain the previous day. We randomly selected one flower from each of 20 different plants in a population over an area of 15 × 15 m. At 1430 h a wooden skewer was used to trigger all 20 flowers. Each flower was then checked every 2 min to record whether the labellum had returned to the down position as required for pollination.

#### Specificity of the pollination system

*How many species of fungus gnats are involved?* Thirteen populations of *P. sanguinea* were visited to collect pollinators (Table 2), using two approaches: (1) baiting for 12 periods of 5 min at each site, during which any visitors to the flower were collected; (2)



FIG. 3. Labellum of *Pterostylis sanguinea*. Letters refer to landmarks used when describing the copulatory behaviour of the pollinator on the flower. A, apex; LS, lower section; US, upper section; C, callus.

following observations that gnats are sometimes terminally trapped in *Pterostylis* flowers (Sargent, 1909; Bernhardt, 1995; Retter, 2009), flowers were dissected to check for the presence of gnats and whether or not they were carrying pollen. The floral dissections were done twice, once early and once late in the flowering season ( $n = 19\text{--}90$  flowers per population, 570 flowers in total; Table 2). All gnats collected were placed in 80 % ethanol for subsequent identification and DNA extraction. Any gnats collected carrying pollen were observed under a dissecting microscope (Leica DCF450) to determine the positioning of the pollen on the animal.

To assess the degree of pollinator specificity, we sequenced the mitochondrial CO1 region, a region commonly used for DNA barcoding in Diptera (Meier *et al.*, 2008). A total of 24 gnats were sequenced from the three populations where live gnats were collected (Kings Park, Shenton Park and Star Swamp). DNA was extracted from whole gnats using a modified salt extraction method (Bruford *et al.*, 1998). Briefly, the gnats were added to 250  $\mu\text{l}$  TNES buffer with 30  $\mu\text{l}$  proteinase K (10 mg  $\text{ml}^{-1}$ ), mixed gently and incubated at 55 °C for a minimum of 18 h. The buffer was then transferred to a tube containing 95  $\mu\text{l}$  of 4 M ammonium acetate, mixed, incubated on ice for 20 min, then centrifuged for 20 min at 16 000  $\times g$ . The intact pollinator was returned to storage in 80 % ethanol. Following centrifugation, the supernatant (400  $\mu\text{l}$ ) was transferred to a clean tube and 1 mL of cold 100 % ethanol was added and the tube contents were thoroughly mixed before incubation for at least 18 h to precipitate the DNA. A pellet of DNA was formed by spinning at 16 000  $\times g$  for 20 min. The supernatant was discarded and the DNA pellet was washed with 500  $\mu\text{l}$  of cold 70 % ethanol and allowed to dry before resuspending in 80  $\mu\text{l}$  TE.

Amplification and sequencing followed the methods of Griffith *et al.* (2011). We used the primers of Folmer *et al.* (1994) as recommended by the Consortium for the Barcoding of Life (<http://www.barcoding.si.edu/protocols.html>). Sequences were edited and aligned using Geneious v6.1.6 created by Biomatters Ltd. A phylogenetic analysis was undertaken using the Phyml plugin in Geneious, using a substitution model of GTR + G. To quantify the genetic divergence within morphologically defined gnat species, we calculated the average Kimura 2 parameter (K2P) distances (Kimura, 1980) and the average percentage of varying base pairs using MEGA 5.05 (Tamura *et al.*, 2011).

*Are all populations of Pterostylis sanguinea attractive to the same pollinator species?* To test whether all study populations of *P. sanguinea* attracted the same pollinator, two flowering stems from each population were picked for baiting in the Kings Park gnat population. When possible we collected individuals representing both green and brown colour forms. Picked orchids were artificially presented within the gnat population and left exposed until a gnat arrived at the flower and was observed to attempt copulation. These gnats were then captured and included in the DNA sequencing dataset.

*What is the role of the site of scent release and morphology in sexual attraction?*

*Is pollinator attraction by floral odour or visual cues?* The ability of gnats to locate hidden flowers was investigated by obscuring

flowers in a black plastic screen made from a washed 1.5 L PET bottle. The screen was 15 cm high but open at the top and raised 5 cm off the ground, allowing access to the concealed flowers. Each trial consisted of a 2-min presentation without flowers within the screen, followed by a 2-min presentation with the flowers. The number of gnats approaching the screen and locating the hidden flowers was recorded for 30 trials in bushland in Kings Park.

*Site of production of the sexual attractant.* Following the observation that copulatory behaviour was only ever observed on the labellum, we tested the hypothesis that the labellum is the sole source of the chemical attractant by dissecting flowers. Each trial consisted of the sequential presentation of the flower without the labellum for the first 5 min, followed by simultaneous presentation of both the flower (minus labellum) and the labellum only (each presented pinned on a separate wooden skewer). The number of gnats responding was recorded along with evidence of sexual behaviour. This trial was repeated on five occasions per flower, for a total of nine flowers.

*Does the morphology of the labellum determine the orientation of the pollinator?* Since gnats typically alighted on the labellum facing upwards, we tested whether the orientation of the labellum influenced their behaviour. We removed the labella and affixed them to wooden skewers using UHU All Purpose Adhesive. Labella were either positioned in the natural vertical position or rotated 180°. Trials were undertaken by presenting a labellum to the fungus gnats until a response was achieved. For every response we alternated between the treatments used. We recorded whether each gnat faced upwards, downwards or sideways and whether they exhibited sexual behaviour (bending and/or probing of the abdomen). Each subsequent trial was conducted at least 1 m from the previous location. If no gnats were attracted within 5 min the trial was terminated. This experiment was conducted using labella from six different plants, for a total of 32 observations per treatment. We employed *G*-tests calculated in GenAlEx 6.5 (Peakall and Smouse, 2006, 2012) to compare both the proportion of gnats copulating with the flower and the proportion showing abdomen bending between treatments.

#### *Ecological consequences of deception of fungus gnats*

*The influence of population size and density on fruit set.* Fruit set (percentage of flowers in a population setting fruit) was recorded for 19 populations in 2008 and 2012. Population size ranged from five to 127 plants. The position of all individuals, as measured with a GPS, was uploaded to ArcGIS 9.3.1 and the minimum convex polygon was calculated to determine the area and density of the population. Regression analysis was conducted in PASW statistics 18 (SPSS Inc., 2009) to determine whether there is a relationship between fruit set and plant population size or density. The 2008 and 2012 data sets were analysed separately. As a test for resource-limited fruit set, in 2008 all flowers on six plants were cross-pollinated by hand in 13 populations. Pollen was sourced from plants more than 5 m from the pollen recipient.

## RESULTS

### *Confirmation and description of pollination by sexual deception*

*Confirmation of pollination by sexual deception.* All insects collected at the flowers ( $n = 33$ ) were male fungus gnats belonging to the family Mycetophilidae (genus *Mycomya*). Ten male gnats visiting flowers were observed carrying pollen of *P. sanguinea* (Table 2). Of the nine found dead in flowers, two were carrying pollen of *P. sanguinea* (Fig. 5). Five gnats were observed to remove pollinia following passage through the flower. One pollen-laden gnat was followed through the entire pollination sequence from alighting on the flower, through attempted copulation, labellum triggering, entrapment, pollen transfer and pollen removal, and departure from the flower. These results confirm that males mycetophilids are legitimate pollinators of *P. sanguinea* (Supplementary Data Video).

Of the 135 gnats alighting on the flower, 57.8% exhibited sexual behaviour with the labellum. When attempting copulation with the flower (Fig. 4), gnats regularly probed at the labellum apex (55.2%), the lower section of the labellum surface (51.7%), the upper section of the labellum surface (51.7%) and the labellum callus (37.9%). Attempted copulations lasted on average  $3.5 \pm 0.2$  s (minimum = 1; maximum = 26 s; mode = 2). Males that attempted copulation averaged  $2.9 \pm 0.3$  copulation attempts per flower (minimum = 1; maximum = 10; mode = 1).

*Description of behaviour of the pollinator on the flower.* During behavioural observations, a total of 135 gnats were observed alighting on the study flowers. Due to the small size of this gnat species, it was not possible to accurately judge how many gnats approached the flowers in flight but then flew away without alighting. Similarly, it was difficult to observe gnat behaviour as they arrived at the flowers in flight. However, on the few occasions when the sunlight was at a suitable angle, it was observed that the gnats arrived at the flower after flying 10–30 cm above



FIG. 4. Male fungus gnat (genus *Mycomya*) showing copulatory behaviour with the labellum of *Pterostylis sanguinea*. Photograph by R. D. Phillips.



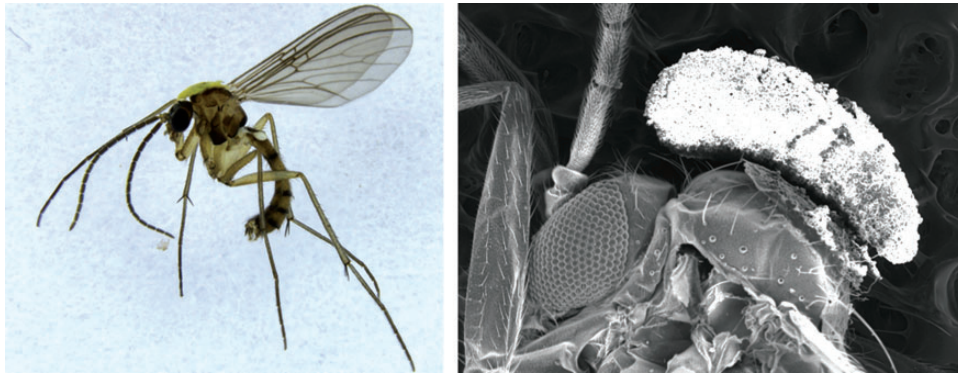


FIG. 5. Male fungus gnat (genus *Mycomya*) showing the position of pollen deposition. Photographs by D. Scaccabarozzi (left) and K. W. Dixon (right).

the ground, in a rapid zig-zag flight. The highest number of gnats arriving at the flower was within the first 30 s after the trial commenced, with visitations gradually decreasing (Fig. 6).

All but two of the 135 gnats observed alighting on the flower landed directly on the labellum. The labellum triggered on 13 occasions, with six triggerings resulting in entrapment. Consequently, only 4.4 % of visitations were likely to lead to contact with the stigma. On the three occasions when triggering of the labellum was filmed, the gnat was either copulating with the labellum apex (twice) or the callus (once) while grasping the labellum and appeared to pull the labellum towards its body. Once inside, gnats were observed flying inside the bulbous portion of the galea, likely bringing them into frequent contact with the stigma. Pollen was evenly distributed over the stigmatic surface ( $n = 10$ ), indicating that the pollen is gradually smeared over the surface rather than deposited as intact pollinia. Gnats were trapped within the flower for between 1 and 7 min (mean =  $226 \pm 63$  s).

On 18 occasions a gnat alighted on a flower when the labellum had already been triggered. In each of these cases the gnat attempted to grasp the labellum. Twice the gnat was observed to reset the labellum by pulling it down. While the gnats began to attempt copulation, the labellum did not trigger. On six occasions the gnats showed at least some copulatory behaviour with the triggered labellum.

*How long does the labellum take to reset?* The labellum showed considerable variation in the length of time to reset, ranging from 6 to 112 min (mean =  $52.5 \pm 7.7$ ,  $n = 20$ ).

#### *Specificity of the pollination system*

*How many species of fungus gnats are sexually attracted?* The taxonomy of fungus gnats in Australia is poorly resolved, with the only substantial taxonomic paper post-1900 being a generic-level revision (Tonnoir, 1929). Colless (1970) estimated that only one-third of the Australian fauna had been described. Using the generic key in Tonnoir (1929), we identified the gnats as a single species of the genus *Mycomya*. However, due to a paucity of relevant literature, we have been unable to identify the gnat at the species level. It should be noted that the revision of Tonnoir (1929) included no specimens from the western half of the continent, suggesting that this is likely to represent an undescribed taxon. Morphological examination suggest that all of the specimens we collected belong to a single species based on consistency of the morphology of the genitalia.

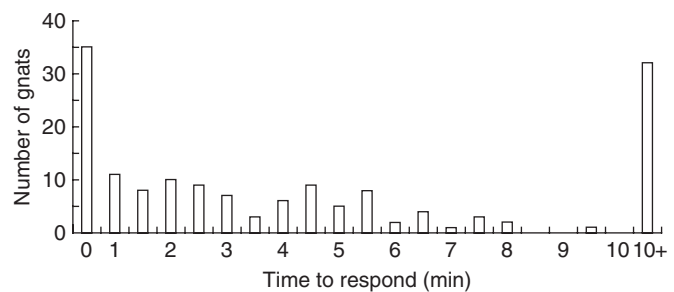


FIG. 6. Time taken by gnats to respond to experimentally presented bait flowers of *Pterostylis sanguinea*. The rapid response is characteristic of some families of insects that are attracted to flowers by the release of floral odours that mimic sex pheromones (e.g. thynnine wasps and ichneumonid wasps).

Sequencing of the mt DNA CO1 region confirmed that all gnats attracted to *P. sanguinea* belong to a single species. All floral visitors formed a clade with 100 % bootstrap support, without any support for internal structure (Supplementary Data Fig. S1). Intraspecific divergence was low, with an average of only 0.8 % sequence divergence using both the KP2 and proportion of base pairs methods. This value is lower than the mean of 1.3 % for 334 species of Diptera reported in Meier et al. (2008). There are no CO1 sequences from other species of *Mycomya* currently available on GenBank, preventing a rigorous comparison of the level of genetic divergence within and among species.

Baiting with picked flowers from eight populations from the Kings Park gnat population showed that specimens from all populations, including both green and brown colour forms, were attractive to the same gnat species.

#### *What is the role of the site of scent release and morphology in sexual attraction?*

*Is pollinator attraction by floral odour or visual cues?* A total of 27 gnats were attracted to orchids concealed within the screen, demonstrating that long-range attraction (> 1 m) is by floral odour. Of the gnats attracted, 18 alighted on the labellum of the concealed orchids. The remaining nine gnats circled the outside of the screen, staying within close proximity for several seconds. No gnats were observed to fly towards the screen when the orchids were absent.



*Site responsible for the production of the sexual attractant.* In the floral dissection experiment, a total of 50 gnats were attracted to the labellum while none were attracted to the remainder of the flower. Of those trials where gnats responded, on average  $2.5 \pm 0.3$  gnats alighted on the labellum per trial. Further, 29 of the 50 gnats showed sexual behaviour with the labellum. All 29 showed bending of the abdomen, with 26 of these also probing at the labellum with the abdomen.

*Does the morphology of the labellum determine the orientation of the pollinator?* All 32 gnats alighting on the dissected labellum glued in its natural vertical position faced vertically upwards. Similarly, 28 out of the 29 gnats landing on the upside-down labella also faced vertically upwards. When the labellum was in its natural position 75% of gnats showed bending of the abdomen, while 71.8% of gnats both probed at the labellum and bent their abdomen. However, when the labellum faced downwards the proportion of gnats showing sexual behaviour was significantly lower, with only 27.6% showing bending of the abdomen ( $G = 14.26$ ;  $P < 0.001$ ) and 20.7% probing at the labellum ( $G = 16.81$ ;  $P < 0.001$ ). Thus the sexual response is strongly influenced by labellum orientation in relation to the gnats' preference for upright landing.

#### *Ecological consequences of deception of fungus gnats*

*Influence of plant population size and density on fruit set.* Across all 19 populations, fruit set averaged  $23.7 \pm 4.4\%$  (s.e.) in 2008 and  $27.7 \pm 3.6\%$  in 2012. Following hand pollination in 2008, fruit set averaged  $82.0 \pm 2.6\%$ , demonstrating that fruit set is primarily limited by pollination. There was no significant relationship between fruit set and either population size or density in 2008 (population size,  $P = 0.410$ ; density,  $P = 0.436$ ) or 2012 (population size,  $P = 0.095$ ; density,  $P = 0.355$ ).

## DISCUSSION

### *Evaluation of sexual deception in *Pterostylis sanguinea**

Following a history of over 100 years of study (e.g. Cheeseman, 1872; Sargent, 1909; Coleman, 1934; Hyett, 1960; Bernhardt, 1995), here we confirm for the first time that pollination by sexual deception occurs in the genus *Pterostylis*, with *P. sanguinea* pollinated solely by sexual deception of male fungus gnats. Our discovery represents the first case of sexual deception involving a member of the Mycetophilidae.

As is typical for most species of sexually deceptive orchid, only one pollinator species was recorded (see Gaskett, 2011, for review). The males appear to show courtship behaviour with the flower in the form of curling of the abdomen, though unlike sciarids (the sister family to Mycetophilidae), the males did not exhibit wing fanning during courtship (Liu et al., 2002; Blanco and Barboza, 2005). However, they regularly engaged in vigorous copulatory probing with the abdomen over the upper surface and apex of the labellum. Thus, *P. sanguinea* meets two of the criteria needed to confirm pollination by sexual deception: pre-mating behaviour and attempted copulation (Table 1).

As observed in other sexually deceptive genera of the Orchidaceae (e.g. Kullenberg, 1973; Stoutamire, 1983; Shiestl et al., 2003; Ayasse et al., 2003), long-range attraction of

pollinators by *P. sanguinea* is achieved by floral odour. However, this contrasts with the fly-pollinated daisy *G. diffusa*, to which male bombyliid flies are attracted to the visual cue of black spots that mimic the appearance of females (Ellis and Johnson, 2010; De Jager and Ellis, 2012). The response of the male gnats to the floral odour of *P. sanguinea* is rapid, with peak numbers arriving in the first minute. This represents an interesting parallel to the sexually deceptive systems involving thynnine wasps, which show a similar response (Peakall, 1990).

While we have confirmed the presence of sexual deception in *P. sanguinea*, we have been unable to ascertain whether ejaculation occurs on the flower. Though rare in sexually deceptive systems, ejaculation on the flower has been confirmed in Australian species of *Cryptostylis*, all of which are pollinated by sexual deception of an ichneumonid wasp (Coleman, 1928; Erickson, 1951; Gaskett et al., 2008). Blanco and Barboza (2005) also provided evidence that ejaculation occurs in the *Lepanthes* pollinated by sciarids. In *Lepanthes*, the pollinator grasps the labellum appendix with genitalic claspers before assuming the tail-to-tail copulatory position (Blanco and Barboza, 2005). By contrast, the pollinators of *P. sanguinea* probe with their abdomen at multiple places over the labellum, without the abdomen ever fixing on a single point. When combined with the rapid triggering of the labellum and subsequent entrapment of the pollinator, this behaviour suggests that ejaculation is unlikely in this pollination system.

### *The importance of labellum morphology for sexual attraction*

While floral odour is critical for long-range attraction, morphological features on the surface of the labellum also appear to be important for sexual attraction to the flower. When the labellum was rotated  $180^\circ$ , the frequency of sexual behaviour greatly declined, suggesting that the texture and shape of the surface of the labellum may be critical for both the positioning of the pollinator (e.g. Kullenberg, 1961) and for stimulating further sexual behaviour. However, the gnats observed attempted to copulate with multiple parts of the labellum surface, suggesting that they are not reliant on a precise morphological cue. An interesting possibility is that the floral scent may be released from a specific part of the labellum which, when combined with appropriate morphology, elicits stronger sexual behaviour from the gnat.

At present, we cannot rule out colour as a possible additional signal leading to sexual attraction. However, to human eyes the colour of the labellum is highly variable between individual orchids, and we did not detect any evidence for differential pollinator behaviour among the floral colour morphs. Unlike the labellum of some sexually deceptive orchids, that of *P. sanguinea* does not appear to bear a close resemblance to the colour of fungus gnats, though this interpretation needs to be supported by spectral reflectance data in concert with a dipteran vision model (e.g. Gaskett and Herberstein, 2010).

### *Comparison of sexual deception in *Pterostylis sanguinea* with that in other orchid genera*

Our detailed quantification of the pollination process for *P. sanguinea* permits comparison of the efficiency of the pollination process with that of other sexually deceptive genera.

TABLE 3. Quantification of the behaviour of fungus gnat pollinators on *Pterostylis sanguinea* with data on thynnine wasp-pollinated species (Phillips et al., 2013)

	<i>Pterostylis sanguinea</i> <i>Mycomya</i> sp.		<i>Caladenia pectinata</i> <i>Zaspilothynnus nigripes</i>		<i>Drakaea livida</i> <i>Zaspilothynnus nigripes</i>	
	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>
Alighting	X	X	60.1	233	80.5	313
Touched labellum	99.3	135	66.4	233	98.8	313
Courtship	54.1	133	X	X	X	X
Copulation with labellum	49.6	133	0	233	34.5	313
Labellum triggered	9.6	135	X	X	81.3	313
Potential column contact	4.4	135	3.6	233	42.5	313

In the case of *Drakaea livida* the hinge in the labellum was flipped through the momentum of the pollinator rather than actively moving.  
X, not applicable or not recorded for that species.

Compared with the thynnine wasp-pollinated *D. livida*, *P. sanguinea* has a very low rate of converting pollinator attraction into potential column contact (Phillips et al., 2013) (Table 3). While in *P. sanguinea* the proportion of pollinators alighting on the labellum and attempting copulation is very high, the inefficiency occurs with the low incidence of labellum triggering. It may be that only particularly large or particularly vigorous gnats succeed in triggering the labellum. Alternatively, the low frequency of the labellum triggering could be an artefact of using bait flowers. When wild plants are touched by the observer, the labellum of most flowers triggers immediately. By contrast, we observed that bait flowers tended to be less sensitive after they reset, following their initial triggering when picked.

#### Evolution of sexual deception in *Pterostylis*

Of the over 400 species of *Pterostylis*, a large number are likely to be pollinated by sexual deception. Several large species groups, which form a clade (Janes et al., 2010; Clements et al., 2011), share very similar floral morphology with *P. sanguinea*, all being characterized by an insectiform labellum presented in front of deflexed lateral sepals (Janes et al., 2010). Evidence from other lineages of sexually deceptive orchids suggests that this pollination strategy is characterized by rapid speciation through pollinator-mediated isolation (Cozzolino and Widmer, 2005; Peakall et al., 2010). If this is the case, the evolution of pollination by sexual deception could have driven the large radiation of species evident in some groups of *Pterostylis*.

Many Australian orchids possess labella that are connected by a short hinge that allows some movement during pollination. However, our confirmation of pollination by sexual deception in *P. sanguinea* strengthens an interesting trend among Australian sexually deceptive orchids, where many species have evolved touch-sensitive, motile or moveable labella. For example, *Paracaleana* and *Caleana* use a hinged trigger mechanism to trap pollinators (Hopper and Brown, 2006). Similarly, *Arthrochilus*, *Drakaea*, *Spiculaea* and some species of sexually deceptive *Caladenia* exhibit highly developed hinged labella (Peakall, 1990; Alcock, 2000; Jones, 2006; Phillips et al., 2009), which appear to aid in the precise positioning of the insect for pollination (Phillips et al., 2013). We hypothesize that the evolution of such precise motile or moveable labella is

more likely in sexually deceptive systems because (1) sexually deceived animals are more vigorous with the flower, increasing the effectiveness of a trigger or hinge mechanism, (2) the extreme pollinator specificity means that mechanisms reliant on a specific body size or weight of pollinator are more likely to evolve, and (3) sexually deceived visitors are strongly attracted to the site of scent release, creating the opportunity for this specific positioning to be exploited.

While it is likely that many of the *Pterostylis* with similar morphology to *P. sanguinea* are using sexual deception, the pollination system of the full diversity of floral forms in the genus remains a mystery. In the species groups that have upswep lateral sepals forming urn-shaped flowers, the labellum is often entirely concealed within the flower. The obscured labellum suggests that if sexual deception is involved the pollinator must be lured into the flower by scent and then trapped rather than relying on an insectiform labellum to position the pollinator. Alternatively, these groups may be attracting pollinators as a shelter site or some other strategy (Adams and Lawson, 1993). *Pterostylis* appears to be a notable contrast with most other sexually deceptive species, which have evolved from lineages primarily pollinated by food-foraging hymenoptera (Steiner et al., 1994; Kores et al., 2001; Cozzolino and Widmer, 2005; Phillips et al., 2009). The intriguing morphology and evolutionary history of *Pterostylis* suggests that if more pollination systems can be resolved, *Pterostylis* will yield novel insights into the evolution of pollination by sexual deception.

#### Ecological consequences of deception of fungus gnats

Fruit set in *P. sanguinea* is higher than reported for most species of sexually deceptive orchid (references in Gaskett, 2011). Further, following investigation of 19 populations, *P. sanguinea* maintained a similar level of fruit set across all population sizes and densities. In the sexually deceptive *Drakaea glyptodon*, which is pollinated by a single species of thynnine wasp (Peakall, 1990), pollination rates were highest in small populations (fewer than ten plants; Phillips, 2010). Thus, maintaining frequent fruit set in small populations may represent an advantage of sexual deception over pollination by food-foraging insects, which in many cases show a decrease in pollination rate at small population sizes (e.g. Ghazoul, 2005;

Jacquemyn *et al.*, 2007; Brys *et al.*, 2008; Johnson *et al.*, 2009; Duffy *et al.*, 2013; though see Johnson *et al.*, 2012). In the case of sexually deceptive systems, it may be that the chemical attractant causes pollinators to seek out orchids even when they occur in low numbers or at low density, thereby avoiding the negative consequences of small population size (Peakall and Beattie, 1996).

Should sexual deception prove to be widespread in *Pterostylis*, this will have important consequences for the conservation of many species. In south-western Australia, sexually deceptive orchids are characterized by a high incidence of rarity, most likely as a by-product of a specialized pollination strategy (Phillips *et al.*, 2011). Studies of extremely specialized pollination systems have confirmed that these plants are highly vulnerable to a decline in the pollinator species, leading to reduced fecundity (Anderson *et al.*, 2011; Pauw and Bond, 2011; Pauw and Hawkins, 2011). In undisturbed landscapes, natural scarcity of the pollinator can contribute to rarity of the orchid through reducing the availability of suitable habitat (Phillips, 2010). While these studies suggest a need to understand the ecology of the pollinators of *Pterostylis*, lack of knowledge on the ecology and taxonomy of fungus gnats may represent an impediment to effective conservation efforts.

### Conclusions

Here we confirm for the first time that pollination by sexual deception operates in *Pterostylis*. Our discovery also represents the first known case of the sexual deception of the Mycetophilidae. While remarkable inroads are being made into the pollination biology of some groups of sexually deceptive orchids, this discovery in *Pterostylis* highlights the prospect that many future discoveries of this pollination strategy are still possible, both within and beyond the Orchidaceae. In particular, an expansion of natural history studies with a focus on the large radiation of tropical orchids pollinated by Diptera is likely to yield other candidate systems. However, a clearer understanding of the evolution of this intriguing pollination strategy and its ecological consequences will be best achieved by integrated studies that combine the fields of ecology, genetics, phylogeny and chemical ecology (Peakall *et al.*, 2010; Xu *et al.*, 2011; Peakall and Whitehead, 2014). In the light of the likely large differences in chemical communication, mating systems and sexual behaviour between Diptera and Hymenoptera, future multidisciplinary studies of *Pterostylis* and other fly-pollinated genera promise to yield many novel insights into the ecology and evolution of sexually deceptive systems.

### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Figure S1: results of DNA barcoding of pollinators. Video: showing a fungus gnat attempting copulation with the labellum of *Pterostylis*, being trapped in the flower and escaping with pollen attached to its thorax. The fungus gnat seen on the left shows the behaviour observed in the vast majority of gnats, where the animal faced vertically upwards during attempted copulation.

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