

Persistence through
resilience:

*How sexually deceptive
orchids make the most of
their pollinators.*

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Cryptostylis subulata and its sexually deceived pollinator, *Lissopimpla excelsa*. Photo by Callum Young. Kaimaumu, New Zealand.



For my parents,

Thank you for teaching me to ask *Why?*

(even if it got old fast)



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Abstract

Exploitation of co-operative relationships is found widely in nature and deception can impose acute fitness costs (including death or reproductive loss), so how do these relationships persist? The Australasian Tongue Orchids, *Cryptostylis* spp. are extreme deceivers: they achieve outstanding pollination rates, eliciting ejaculation from their male wasp pollinator, *Lissopimpla excelsa*. Here, I use this system to examine individual-level costs and responses to exploitation through field experiments and evaluate population-level responses to sexual deceit using mathematical modelling and a survey of museum and digital records.

In my field experiments, I quantify the costs of sperm for orchid pollinators, finding males may become depleted in their lifetime. I also find evidence of localised morphological and behavioural changes in response to orchid deceit. Compared to the same insect species in areas without orchids, male pollinators in areas with orchids have longer antennae, take longer to arrive at an orchid, have shorter orchid mating durations, and have smaller ejaculates. Antennae length does not appear to be a counter-adaptation to deception as it does not confer discriminatory ability.

Instead, males with longer antennae had faster response times and were more likely to pollinate orchids. I hypothesise that rather than counter-adaptations, by manipulating population sex-ratios, *Cryptostylis* orchids enhance the effects of scramble competition in this species: creating males that more readily search for, and pollinate, these orchids.

Mathematical modelling shows that a putative 'resilience' trait, haplodiploidy (in which females can reproduce without sperm, albeit only sons), helps exploited pollinators persist when experiencing sperm depletion. Museum and digital records corroborate this finding. I present two models, one from an orchid's and one from a pollinator's perspective. I found that orchids exploiting haplodiploids receive a double advantage: a pollinator resilient to reproductive interference (haplodiploid populations were less likely to become extinct than diploids and had improved female production); and an enhanced supply of males to act as pollinators (orchids that target haplodiploid populations had higher pollination rates than if they were to target diploids). Combined, these findings present a novel mechanism to explain the persistence of costly relationships: *resilience*. The idea that certain pre-existing life-history traits allow pollinators to cope with exploitation while improving long-term exploiter success.

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Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. **Please include one copy of this form for each co-authored work.** Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 4: 'Orchid sexual deceit affects pollinator sperm transfer.'

Nature of contribution by PhD candidate	Wrote manuscript, designed experiments and performed all field work, carried out statistical analyses, responded to reviewer comments	
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Certification by Co-Authors

The undersigned hereby certify that:

- ❖ the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- ❖ that the candidate wrote all or the majority of the text.

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Introduction and thesis outline

Ecosystems are moulded by the interactions and relationships between its organisms: from facilitative, to neutral, to antagonistic (Hui, Minoarivelo, Nuwagaba and Ramanantoanina, 2015; Price, 2002; Soler, 2014). Many of these relationships are the consequence of coevolution, involving reciprocal change between interacting species, which drives the natural selection of traits that either maintain or increase an individual's fitness (Brockhurst and Koskella, 2013; Hembry, Yoder and Goodman, 2014). The most intuitive co-evolutionary relationships are those that are mutually beneficial (Bronstein, 2001): reciprocal interactions between members of different species that provide quantifiable benefits to each partner.

Nevertheless, many relationships are asymmetric where only one partner benefits, at a cost to the other. For instance, sexual cannibalism (Barry, Holwell and Herberstein, 2008); parasitism (of sperm, broods, or that which results in disease; or even conflict between sexes (Bro-Jørgensen, 2007; Lauer, Sih and Krupa, 1996)). When the costs are high, what allows exploited populations to persist?

To date, three key hypotheses address this. First, arms races (Feeney, 2017; Wong and Schiestl, 2002) – in which victims of a costly interaction defend themselves against exploitation with some trait and then their defences select for counter-adaptations in the exploiter. For instance, in avian brood parasitism, hosts will resist parasitism through mobbing and parasites will respond with cryptic plumage or behaviour (Feeney, Welbergen and Langmore, 2012). Similarly, the evolutionary lag hypothesis suggests that persistence is explained because we cannot observe counter-adaptations arising from, or extinction of, the exploitative relationship. This is because either there has not been enough time to evolve a defence or for the relationship to go extinct; or the exploited group do not have the necessary genetic variation to evolve a defence (Davies and Brooks, 1989).

Second, indirect fitness benefits that compensate for costs exerted upon the exploited party. This occurs in praying mantids, where cannibalistic females substantially improve their body condition by consuming courting males before, during or immediately after mating (Barry, Holwell and Herberstein, 2008). Despite males' loss of life and subsequent fitness impacts, the relationship is maintained because of indirect fitness benefits. Cannibalised males typically have more offspring and improved offspring

survival, as they have improved their mate's condition (Barry et al., 2008; Berning et al., 2012; Welke and Schneider, 2012). Finally, the persistence of costly relationships can be intuitively explained by the idea that low encounter rates allow populations to persist despite exploitation or conflict (Kokko and Rankin, 2006; Kokko et al., 2008).

These mechanisms do not, however, adequately explain several relationships. For instance, sexually deceptive orchids – pollinated by male insects fooled into mating with flowers that mimic the sex pheromones and appearance of females (Ayasse, Stökl and Francke 2011; Gaskett 2012) – can have very high fitness, while imposing potentially acute costs on their pollinators (Gaskett, Winnick and Herberstein 2008; Wong and Schiestl 2002). Deceptive orchids never offer rewards to their pollinators and have very high pollination rates – so we cannot explain their success through indirect fitness benefits or low encounter rates (e.g. as per Lehtonen and Whitehead, 2014). Moreover, there is presently no evidence for counter-adaptations in response to deception, despite the suggested costs of deceit.

This thesis investigates the persistence of sexual deceivers and their pollinators, with a focus on the Australian and New Zealand tongue orchids, *Cryptostylis* spp., and their pollinator, *Lissopimpla excelsa*. I

combine mathematical modelling, field experiments and museum surveys to quantify the individual-level costs of, and responses to, sexual deceit. I also propose a new hypothesis to explain the persistence of costly relationships: 'resilience', by exploring population-level responses over evolutionary time through mathematical modelling. The chapters have been written for publication and follow the formatting preferences of each journal.

Chapter 1 is a review of our current understanding of the evolutionary maintenance of costly relationships, focusing on sexual deception. In addition, this review highlights a new approach that focuses on the life-history of exploited species and explores *resilience* as an alternative hypothesis to explain how asymmetric pairings persist. This paper has reached the final round of the *Austral Ecology* journal's award for best Early Career review.

Chapter 2 is a summary of this thesis, now in review for *Nature*. It presents a snapshot of my findings here: the behavioural and morphological responses of *L. excelsa* in response to deception; modelling the impacts of haplodiploidy on population persistence and testing the outcomes of that model with museum surveys. It is my intention to first

try to publish this manuscript, and then if it is not accepted – separate into several smaller manuscripts (chapters 3, 5 and 6).

Chapter 3 uses field experiments to test whether counter-adaptations arise in response to sexual deception. Acknowledging the impact orchids might have on pollinator population, I assess whether any changes I observed in response to deception are a result of counter-adaptations or, indeed, a consequence of a male-biased sex ratio: increased effects of scramble competition. This paper is formatted for *Functional Ecology*.

Chapter 4 experimentally quantifies the costs of *Cryptostylis* orchids sexual deceit for its pollinator, wasps of *L. excelsa*. Here, I establish the number of sperm the pollinator ejaculates on deceptive orchid flowers, and test if this duped wasp experiences sperm limitation, and whether this impacts his ejaculate size after deception by an orchid. Sperm limitation has implications for males' future and lifetime reproductive success, and consequently, the population dynamics of the pollinator species, and therefore the ultimate fitness of the orchid, and the evolutionary maintenance of orchid deception systems in general. This paper is published in *Functional Ecology*.

Chapter 5 uses a mathematical model to test whether haplodiploidy confers some resilience to pollinators deceived by sexually deceptive orchids. It provides evidence that pollinators are better able to cope with the costs of sexual deception (in terms of persistence, and overall reproductive output) if they are haplodiploid. This paper is formatted for *Proceedings of the Royal Society of London, Series B*.

Chapter 6 tests the predictions of this model through a survey of museum and digital records. It tests the hypothesis that, by eliciting sperm wastage, sexually deceptive orchids create a male-biased population of pollinators: enough to fertilise females and pollinate orchids and maintain both populations. This paper is formatted for *The Journal of Natural History*.

Chapter 7 builds on my first mathematical model to determine whether it is advantageous for orchids to target haplodiploid pollinators. I find that sexually deceptive orchids that target haplodiploid pollinators obtain higher pollination rates than if they were to target diploid pollinators. This paper is formatted for *Proceedings of the Royal Society of London, Series B*.

Chapter 8 is a conclusion that discusses how this thesis contributes to the field of evolutionary ecology. The findings of my research show that it is crucial to consider the perspective of exploited parties when assessing deceived-duped dynamics of sexual deception.

All data from this thesis is archived with my supervisor, Dr. Anne Gaskett.

I hope this thesis can offer a fresh perspective on orchid-pollinator relationships and evolution, as well as ideas for future studies of antagonistic co-evolutionary relationships.

Chapter 1: Resilience – how exploited species persist?

In review in Austral Ecology

1.1 Introduction

Life histories and evolutionary trajectories of all species can change due to other species with which they share their environment. Coevolution, in which species evolve reciprocally by exerting selection pressures on one-another, can shape these trajectories (Price, 2003; Soler, 2014; Hui et al., 2015; Bronstein, 2001), and drive the selection of traits that maintain or increase an individual's fitness (Brockhurst and Koskella, 2013; Hembry et al., 2014). Co-evolutionary systems include mutualistic or symbiotic relationships, but also relationships where species that successfully gain an advantage over others can secure more food, improved reproduction or better avoid predators (e.g. hosts and parasites or predators and prey; Mokkonen and Lindstedt, 2016).

Exploitation can occur through deceit. Across a wide range of ecological interactions and taxa, individuals deceive others by exploiting their perception, and gain fitness benefits (Mokkonen and Lindstedt, 2016; Font, 2018). For instance, to hunt spiders, assassin bugs will imitate the vibrations of trapped insects in webs (Wignall and Taylor, 2011); predatory praying mantises mimic flowers to lure pollinators as prey (O'Hanlon et al.,

2014); brood-parasitic cuckoos lay eggs that mimic the colour of their host eggs (Yang et al., 2017); quorum sensing bacteria will signal for the production of 'common goods' without producing it themselves (Katzianer et al., 2015; Diggle et al., 2007; Czárán and Hoekstra, 2009); and sexually deceptive orchids mimic insect mating signals to fool male insects into mating with, and pollinating their flowers (Gaskett et al., 2008). These deceptive interactions have at least one thing in common: they subject the 'duped' individuals to some cost. These costs may be negligible (e.g. energy, time; Wong and Schiestl, 2002) or larger (e.g. food or a mating event; Brunton Martin et al., 2020), and may even result in an individual's death (Barry et al., 2008).

The evolutionary maintenance of mutualistic relationships is intuitive – if both parties benefit, there is strong selection for mutualisms or factors that ensure the relationship remains mutualistic. Indeed, the most compelling evidence for coevolution or cospeciation arises from research on mutualistic relationships (Vienne et al., 2013). In contrast, the relationships that impose costs on one partner are harder to explain (Kokko and Brooks, 2003). Theory predicts that deceptive relationships break down due to the extinction, decline or evolved resistance of the exploited partner (Hesse and Buckling, 2016; Vitale and Best, 2019; Ricklefs, 2010; Gibson et al., 2010). When relationships persist in nature, this may be a consequence of a variety of mechanisms, which I explore in this review. In addition to coevolution (Feeney, 2017), studies explain the maintenance of exploitative interactions through indirect fitness benefits accruing to the

exploited partner (Sardell et al., 2012; Barry et al., 2008) or low encounter rates that mean there is only weak selection on the exploited partner (Kokko and Rankin 2006; Kokko et al., 2008).

Orchids provide an overlooked opportunity for studying exploitation and mechanisms of persistence. While many orchids are rewarding, the Orchidaceae has the largest number of deceptive species. Approximately 80% of all deceptive angiosperms are orchids, and one-third of orchids are thought to be deceptive (Jersáková et al., 2006). Shrestha et al., 2(2020), however, recently found that this might not necessarily be the case, and many orchids previously considered rewardless may actually have rewards – even if very small rewards. Hence, new work on these systems should aim to verify rewardlessness.

Deceptive orchids frequently exploit their pollinators' behaviour (typically foraging and reproductive behaviours of pollinators; Table 1), to their benefit. They do not offer any reward in return, and can harm their pollinator (Wong and Schiestl, 2002; Brunton Martin et al., 2020). If deception harms the pollinator, I might expect the presence or evolution of mechanisms to allow the pollination strategy to persist. For example, food deceptive orchids can be deceptive through batesian floral mimicry (where orchids mimic a specific rewarding model, exploiting a conditioned response of pollinators) or generalised food deception (where orchids advertise general signals of food, exploiting innate or learned responses of pollinators; Jersáková et al., 2006). While both strategies likely rely on low encounter rates so that pollinators do not learn to avoid orchids,

batesian mimics probably rely on being rare relative to the number of rewarding models in their area. In contrast, generalised food deceptive models rely on being a rare morph due to polymorphisms in colour or scent (Jersáková et al., 2009, but see "Maintenance through low encounter rates" in this review).

Orchid deception usually targets foraging and reproductive behaviours of pollinators, but the costs to pollinators are often small (Gaskett, 2011; Schiestl, 2005, Table 1). The mechanisms behind maintenance of these systems likely rely on low encounter rates (Mokkonen and Lindstedt, 2016). However, more research is needed in this area to quantify true encounter rates between pollinators and orchids.

Indeed, many hypothesised mechanisms do not adequately explain the persistence of costly relationships, such as sexual deception by orchids. Sexual deception is an extreme and atypical form of pollination, in which flowers will lure a male pollinator with duplicitous sex signals. Fooled males will then mate with the flower and unintentionally collect or deliver pollinia (Schiestl, 2005). In some cases, males will ejaculate and waste sperm on the orchid (Gaskett et al., 2008; Brunton Martin et al., 2020; Blanco and Barboza, 2005). There are currently 28,000 accepted species of orchid worldwide – approximately 400 of these are sexually deceptive (Gaskett, 2011). Sexual deception is a surprisingly common strategy in orchids, given that it likely exerts a direct cost on pollinator fitness.

Sexual deception could impose costs on pollinators in various ways. It can harm male pollinators by causing missed mating opportunities with real females and wasted ejaculate (Wong et al., 2004; Jager and Ellis, 2014; Wong and Schiestl, 2002; Brunton Martin et al., 2020). Females of the pollinator species are also likely to suffer costs as they must compete with orchids for mates in both space and time as their mating season coincides with orchid flowering season, and the orchids only occur where wasp populations are present (Gaskett and Herberstein, 2006; Brunton Martin, 2020). For example, male wasp pollinators of sexually deceptive *Chiloglottis* orchids (*Neozeleboria cryptoides*, Family: Tiphidae) are unable to distinguish between orchids and females. Indeed, males find females less attractive when females are among orchids (Wong and Schiestl, 2002), and these flightless females need to move far away from orchids, or they will miss out on mating and reproduction (Wong et al., 2004). Mating events are crucial for female *N. cryptoides*: they rely on males to provide them with not just sperm, but also food and transport to oviposition sites. If a male chooses an orchid over a female, he reduces female foraging and reproductive fitness, which in turn likely impacts the pollinator's broader population dynamics.

Costs associated with sperm wastage may be particularly acute when an orchid visit triggers pollinator ejaculation. Sexually deceptive *Cryptostylis* orchids exploit males of the solitary parasitoid species *Lissopimpla excelsa* (Family: Ichneumonidae, subfamily: Pimplinae, Figure 1). These orchids frequently trick males into mating with and

ejaculating on, the flower during pollination (Gaskett et al., 2008; Brunton Martin et al., 2020; chapter 4). Parasitoid species, in particular, are likely to become permanently or temporarily sperm depleted after several mating events (Jager and Ellis, 2014; Olsson et al., 1997; Boivin et al., 2005; Damiens and Boivin, 2006; Boulton et al., 2015).

Therefore, for these males, wasting sperm on an orchid may well be an acute cost. Sperm production and limitation, however, is a reasonably poorly studied area. To date, there is only one study that estimates sperm production in an orchid pollinator: males of *L. excelsa* became at least temporarily sperm depleted during mating with sexually deceptive orchids (Brunton Martin et al., 2020, chapter 4). The same study found that naïve males deposit significantly less sperm than experienced males, suggesting that *L. excelsa* may vary their ejaculate size in response to the false signals produced by orchids, or in response to wasps' perception of the abundance of 'females' (either true female insects, or orchids).

Discussions of sexual deception often dismiss the costs as benign and insufficient to exert selective pressure on pollinators. However, there is presently no empirical evidence for lack of evolutionary change in the pollinator, nor experimental evidence for costs (or lack thereof). Coevolution may be a possible explanation for the persistence of sexual deceit, through an arms race between sexually deceptive orchids and their pollinators (Wong and Schiestl, 2002). This arms race would be similar to the quintessential relationship that exists between cuckoos and their unwitting hosts (Krüger, 2007), where an

adaptation in one species (e.g. mimicry of host eggs) changes the selection pressure on another (e.g. improved recognition and rejection in host species). Despite the general reluctance to consider coevolution in deceptive orchid pollination, there is strong evidence for coevolution between rewarding orchids and their pollinators (Boberg et al., 2014; Anderson and Johnson, 2007). Darwin's hypothesis of co-evolution between the Madagascan star orchid, *Angraecum sesquipedale*, and its extremely long-tongued pollinator are a classic and well-supported example (Arditti et al., 2012; Darwin, 1862). Given the incidence of coevolution in rewarding orchids, and in other deceptive plant-pollinator relationships (e.g. in fig-fig wasp interactions; Wang et al., 2010), a reciprocal change resulting from sexual deception could be likely. Experimental tests are lacking here, however, and research in this field is important to determine whether costs imposed by orchids are enough to drive co-evolutionary change between orchids and their pollinators. Counter-adaptations in response to sexual deception might include improved sensory structures (e.g. eyes or antennae) in order to improve discrimination between deceptive orchids and females. If sperm loss is a cost to deceived males, one might also expect increased sperm capacity (larger sperm-storage organs or greater sperm production).

In this review, I explore how relationships persist when there are high costs, with a focus on sexual deceit. First, I outline the origins and evolution of sexual deception and its implications for the maintenance of exploitation. Second, I discuss known mechanisms

that could maintain asymmetric relationships in the context of deception, as well as the likelihood of a co-evolutionary arms race occurring. Finally, as there is not sufficient evidence to explain the success of sexual deceivers, I present a new mechanism, *resilience*. I emphasise the effect that pollinator behaviour, life history, and mating systems have on deceived population persistence. I propose that this resilience is a mechanism that may help account for the persistence of extreme sexually deceptive relationships and contribute to our understanding of the maintenance of other costly, antagonistic relationships.

1.2 Evolution of sexual deception

Sexual deception is considered a derived trait among orchids (Bateman et al., 2003; but see Gang, 2005). Largely, it seems that sexual deception evolved from food deception: shown by research on the large, European genus *Ophrys*, and *Orchis galilaea* (Cozzolino et al., 2001). Some species today exploit both the foraging and sexual behaviour of pollinators (e.g. *Diuris*, *Caladenia*, *Cymbidium*, *Disa* and *Orchis*) and could be considered transitional species under this proposed mechanism of evolution (Jersáková et al., 2006; Cozzolino and Widmer, 2005). However, the direction of evolutionary change between rewarding orchids (generalised), food deception (generalised-specialised) and sexual deception (specialised, see Figure 3B) remains unresolved, in part because two of three primitive orchid orders contain mostly deceptive species (Figure 3A). Food

deception, in particular, might be ancestral in orchids, evidenced in several studies of European and Australian orchids (Orchidinae and Diuridae) – where nectar rewards evolved multiple times in the Orchidinae, Deseae and Diuridae lineages (Cozzolino et al., 2001; Johnson et al., 1998, 2013; Inda et al., 2012; Smithson, 2009; Bell et al., 2009). In contrast, in the higher orchids, Epidendreae, food deception likely evolved from rewarding orchids (Cardoso-Gustavson et al., 2018).

The evolutionary origins of orchid deception have interesting implications for its maintenance. Evidence for co-evolution, i.e. long-term reciprocal change, could be derived by examining if orchid lineages become more specialised over time as pollinator species evolve defences against and in response to losses incurred by exploitation. In orchids, I have found that the degree of specialisation relates to the type of pollination strategy (Figure 3B). Often, increased specialisation with speciation suggests that interspecific interactions and co-evolution drive species divergence (Thompson et al., 2017; Vienne et al., 2013). As genetic constraints often prevent single populations from maintaining numerous alternative phylogenetic states (Smith, 2010), theory predicts that exploitative orchids will become more specialised the longer they are in contact with their target pollinator (Rothstein et al., 2002). Even though the ancestral state of orchids is still unclear, current evidence suggests that sexual deception, which is highly specialised, arises from the less specialised food deception, providing some support for this hypothesis. The alternative outcome, in which orchids trajectories resolve as

becoming more generalised, would instead indicate that exploiters' ecological success and habitat are more important than co-evolution (Vienne et al., 2013; Rothstein et al., 2002). For instance, ecological innovations (such as establishing in a new area) in a specialist orchid species might create a selection pressure to expand its range of pollinators (Thompson, 2009). This seems unlikely in the context of Orchidaceae, as research has never found that more generalised systems (food deception and rewarding pollination) evolve from sexual deception (Cozzolino and Widmer, 2005). Research that focuses on phylogenetic tracking of the Orchidaceae and exploration of pollinator switching associated with environmental changes should improve our understanding of these evolutionary patterns and provide insight on a long-term evolutionary timescale into whether sexually deceptive relationships are co-evolutionary.

1.3 Maintenance through low encounter rates

One of the most intuitive mechanisms for the persistence of costly relationships is simply the idea that low encounter rates allow populations to persist despite exploitation or conflict (Kokko and Rankin 2006; Kokko et al., 2008). That is, the less an exploited group encounters the exploiter, the less of an impact that exploiter has on the population – reducing the chance for learning or counter-adaptation and allowing the deceptive relationship to persist. For orchids, low encounter rates involve the relationship between deceptive orchid density, pollinator learning and pollination success.

Few studies test this, and there is mixed evidence to show that there is a benefit to being rare. In one food deceptive orchid, *Dactylorhiza sambucina*, rare colour morphs had a reproductive advantage (Gigord et al., 2001) – but in several other studies, this was not the case (Pellegrino et al., 2005; Tremblay and Ackerman, 2007; Juillet and Scopece, 2010). In extreme deceivers, such as *Cryptostylis* orchids, it is unlikely that they have low encounter rates as the orchids can be highly abundant, and their pollination rates are extraordinarily high, suggesting frequent interactions with the pollinator. These rates can be up to 90% with a mean of 70%: (Gaskett, 2006; Schiestl et al., 2004); compared to a 30% world average for sexually deceptive orchids from Europe, and temperate/tropical southern hemisphere (Neiland and Wilcock, 1998).

For orchids and their pollinators, low encounter rates could arise because pollinators quickly learn to avoid orchids, a mechanism that may even be accelerated when orchids are highly abundant. For example, a few studies suggest pollinator avoidance learning in response to sexual deception (Wong and Schiestl, 2002; Jersáková et al., 2006; Schiestl, 2005; Paulus, 2006; Weinstein et al., 2016), even in the extreme deceiver *Cryptostylis* (Gaskett et al., 2008). However, avoidance of orchids is not necessarily a learned behaviour in response to orchids. If I consider the pre-existing behaviour of the pollinators of these orchids (entirely solitary parasitoid wasps), these studies may instead be revealing behaviours relating to pollinators' pre-existing mating systems.

Most sexually deceptive orchids are pollinated by solitary bees and wasps, especially parasitoids (Gaskett, 2011), which typically have a mating system based on females releasing sex pheromones to attract a male and mating just once (monandry), and intense scramble competition to respond quickly to a female's signal and be the first to mate with her (Quicke, 2014). Thus, there is likely to be selection to avoid remating with the same female and therefore maximise their number of matings with virgin females. For example, when solitary male parasitoids have found and mated with a virgin female they usually will not return to that site – even if a new virgin female appears (Goh and Morse, 2010; Quicke, 2014). There may be some delay before the site can become attractive again, or maybe the same male can never be attracted to the same site, and only different males will respond. This phenomenon could explain why sexually deceptive orchids that have multiple flowers typically sequentially open each flower along the raceme, rather than opening all the flowers at once for a larger floral display as is typical of rewarding flowers (Arditti, 2002). Not only does this prolong the flowering season, but might also allow for a period where the flowers are not producing any signal, reducing pollinator avoidance by resetting the potential attractiveness of the site. Choice experiments that compare the presentation of a true female with the presentation of a deceptive orchid might show whether true learned avoidance of orchids, or avoidance of a location, takes place;. However, this is challenging as females are often difficult to find (Naumann, 1991), and females emit sex pheromones immediately upon emergence and

quickly attract males, so virgins are almost impossible to source from wild populations (Ridley, 1993; Sandanayaka et al., 2011).

1.4 Maintenance through indirect fitness benefits

Exploitation may also persist when indirect fitness benefits compensate for the costs to the exploited party (Sardell et al., 2012; Dimitriu et al., 2016). For instance, when male-killing bacteria parasitise lady-bird hosts, infected female lady-birds experience decreased oviposition, lower fecundity, higher infertility, shorter adult life-span and death of sons (Majerus et al., 2000; Elnagdy et al., 2011). However, these costs are offset by the improved fitness their daughters experience through sibling-egg cannibalism. An egg meal not only has substantial nutritional value, but also frees up resources for daughters through resource reallocation (Elnagdy et al., 2011).

Similarly, indirect benefits are obtained by male praying mantids, because although sexual cannibalism kills males, it provides them with paternity benefits. Cannibalistic females substantially improve their body condition by consuming courting males before, during or immediately after mating (Barry et al., 2008). Becoming lunch, of course, comes at a cost to the courting male. Nevertheless, males indirectly benefit through increased fertilisation success and improved offspring survival (Welke and Schneider, 2012). No studies have found that deceptive orchids offer any indirect benefits to any pollinators (Table 1). Hence, it is highly unlikely that sexual deception persists due to

this mechanism – there are certainly no rewards they offer that would outweigh the costs of reproductive loss.

1.5 Maintenance through co-evolution

A likely mechanism for the maintenance of sexual deception is co-evolution. Under this mechanism, deception would select for traits that improve the exploited species fitness, and then these defences select for counter-adaptations in the deceiver. Cuckoo brood parasites and their hosts are a classic example of an arms race. Exploitation selects for host recognition of parasitic eggs and mobbing of parasites. In turn, this selects for cuckoo cryptic plumage and behaviour, and eggs that match those of their hosts (Feeney, 2017; Marchetti, 2000; Spottiswoode and Stevens, 2011). Typically, resistance or tolerance are key concepts that reduce costs associated with arms races (Svensson and Råberg, 2010). In brood parasitism, resistance minimises the number of successful exploitations – as would be achieved by mobbing and rejection of parasite eggs.

Resistance can drive the deceptive relationship to extinction (Vienne et al., 2013).

Tolerance, on the other hand, reduces the fitness impact of exploitation. In brood parasitism, the host can accept the parasite but adjust certain traits to minimise the costs of parasitism. For instance, Eurasian magpie (*Pica pica*) rear their young alongside brood parasites and minimise the loss of their young by increasing clutch size (Soler et al., 2001). Arguably, the strategy of tolerance would not result in a co-evolutionary arms

race because tolerance does not reduce the exploiter's fitness (Svensson and Råberg, 2010; Fornoni, 2011). However, empirical evidence for tolerance is presently lacking – and requires more research to achieve a full understanding (Avilés, 2018; Soler, 2018). Importantly, a co-evolutionary arms race may not facilitate the maintenance of the relationship – as the outcome of antagonistic coevolution is often extinction (via the exploited species becoming extinct or escaping exploitation). Some theoretical models, however, suggest prolonged coexistence is possible (Huang et al., 2017; Hui et al., 2018; Schenk et al., 2020). As outcomes of co-evolutionary arms races are most often unobservable in researchers' lifetimes, many interactions appear to remain at an evolutionary standstill.

Does sexual deception coevolve?

Evidence for pollinator-mediated selection.

Although there are few studies of coevolution between sexually deceptive orchids and their pollinators, it is widely accepted that the orchid species adapt to their pollinator preferences. Floral scent is the trait that has received the most attention. Sexual deception relies primarily on orchids' precise chemical mimicry of the species-specific sex pheromones of the female of the pollinating species (Schiestl, 2005; Bohman et al., 2016; Ayasse et al., 2011), particularly for long-range attraction. In sexually deceptive *Chiloglottis* orchids, pollinator specificity relies on floral odours, and speciation underpins chemical change in scent (Peakall et al., 2010). Changes in floral scent that attract

specific pollinators lead to floral isolation and subsequent speciation. Indeed, scent seems to be the primary pollinator attractant and is essential for pollination by sexual deception. In *Ophrys*, *Chiloglottis*, *Drakea* and *Cryptostylis* orchids, pollinators will readily mate with simplistic dummies spiked with orchid scents (Bohman et al., 2016, 2019).

Sexually deceptive orchids also adapt to match the morphologies of their pollinators to achieve improved mechanical fit for efficient pollination (Newman et al., 2015).

Morphological manipulations of *Chiloglottis* orchids reveals that pollinators reduce copulation duration (and therefore pollen transfer) when pollinating flowers with abnormal (very long or very short) callus-tip distances or shortened labella (de Jager and Peakall 2019, 2016). Similarly, in European *Ophrys* orchids, pollinators are less effective when they interact with flowers that have altered lips (the third petal of the orchid that serves as a landing platform for pollinators) and therefore inadequate gripping points on the flower (Rakosy et al., 2017). Finally, there is some evidence to suggest that colour is important in sexual deception. Sexually deceptive orchids may be under selection to be highly detectable for pollinators (Gaskett et al., 2017; Kelly and Gaskett, 2014; Rakosy et al., 2012) or have colours that match that of female pollinators (Gaskett and Herberstein, 2009). Orchids, therefore, seem quite able to evolve in response to their pollinators and are labile in terms of their morphology, scent and colour.

Evidence for orchid-driven selection

While there is some evidence for co-evolution between food-rewarding flowers and their pollinators (e.g. reciprocal evolution between pollinator's tongues that allow them to consume all of a flower's nectar, and longer floral tubes; Ricklefs, 2010; Bili et al., 2016; Vamosi et al., 2006; Arditti et al., 2012) there is presently no data to suggest pollinators evolve in response to deceptive pollination strategies, and phylogenies suggest that co-evolution is unlikely because of the different branch lengths of pollinator and orchid groups (Mant et al., 2002).

This is perhaps because the relationship between deceptive orchids and their pollinators is highly specialised and deceptive: flowers have strong selection pressure to be species-specific, but pollinators do not face costs large enough to need to avoid deceit and elicit an arms race.

However, sexually deceptive orchids interact with their pollinator's reproduction rather than foraging. If orchid interference is a substantial cost to these pollinators, I may expect co-evolutionary responses in the pollinator's reproductive behaviour or anatomy. I may detect an arms race based on reproductive traits that ultimately underlies the evolutionary relationship between sexually deceptive orchids and their pollinators – much like that observed between cuckoos and their hosts. Analysis of the potential reproductive costs imposed by orchids requires consideration of the mating systems of their pollinators – which are surprisingly similar given the multiple independent

evolutions of sexual deception globally. Most sexually deceived pollinators are from species that have scramble competition (Kai-Fong et al., 2019), where males compete to be the first to find and mate with a female. Females are usually monandrous, so once mated is unlikely to mate again (Boulton et al., 2015). Therefore, any search-time males lose in pursuit of an orchid is a cost and could result in missed mating opportunities. Costs to females may also exist – for instance, in orchid patches, females elicit fewer copulations and approaches from males than when they are alone (Wong and Schiestl, 2002). In some species, such as the Thynnine wasps that pollinate sexually deceptive *Chiloglottis* orchids, females not only rely on males for reproduction, but also for transport to food and adequate oviposition sites.

Both females and males may suffer costs if orchids trigger potentially costly sperm wastage, although confirmed examples are rare: e.g. Australian and New Zealand *Cryptostylis*, that share a single, duped pollinator, *Lissopimpla excelsa* (Gaskett et al., 2008; Brunton Martin et al., 2020). South American *Lepanthes* orchids may also elicit sperm wastage, evidenced by traces of spermatophores on their flowers (Blanco and Barboza, 2005). Sperm production is energetically costly in several insect groups (Bunning et al., 2015; Godwin et al., 2017; Zajitschek et al., 2019; Wagner and Bakare, 2017). Parasitoid wasps (the primary pollinator of sexually deceptive orchids; Gaskett, 2011) often have limited sperm production in their lifetime (Boivin et al., 2005; Damiens and Boivin, 2006; Boulton et al., 2015). Parasitoid females are generally unable to detect

whether males have sperm, and so in mating with a sperm-depleted male, may never receive a full complement of sperm or remain pseudo-virgin for their lifetime (Abe, 2019). If males do replenish sperm, they typically produce fewer spermatozoa at a lower rate and with a higher proportion of infertile sperm with every mating event (Vega-Trejo et al., 2019; Wedell et al., 2002; Pizzari et al., 2008). The procedural challenges of detecting spermatophores on orchids (Bressac et al., 2008) may have led to an underestimation of how common ejaculation is.

At present, there are just a few tantalising suggestions that these types of costs could be sufficient to drive change in a sexually deceived pollinator species. One study has suggested that sexually deceptive orchids interrupt the normal mating behaviour of pollinators (Wong et al., 2004).. In response, selection may act on females to promote learned avoidance of deceptive orchids to improve their chances of reproduction. Another study, comparing male pollinators of a single species from sites with and without natural populations of orchids, found that males in sympatry with sexually deceptive orchids had smaller ejaculates but a similar sperm volume (Brunton Martin et al., 2020, chapter 4). This reduction may be a counteradaptation in response to sperm loss or be a strategic response to the perception of abundant females (and orchids) in the males' habitat (Parker and Pizzari, 2010). For both of these early studies, it is unclear whether these behavioural changes arise through plasticity or counter-adaptation. It would be interesting to determine whether there are any morphological responses in deceived

populations. For instance, since pheromone mimicry is the main source of attraction in orchids (Ayasse et al., 2011; Bohman et al., 2019), future research might focus on differences in the chemosensory structures of pollinators that might indicate counteradaptations to better distinguish orchids from real females.

1.6 An alternative explanation for maintenance

There is presently little evidence to support the three existing hypotheses for the maintenance of sexual deception: low encounter rates, indirect fitness benefits or counteradaptations. Here I present an alternative hypothesis for the maintenance of this relationship: *resilience*. This mechanism purports that life-history traits of individual pollinators buffer the costs of deception, while also making them uniquely suited to it.

The definition of resilience is better understood by comparing it to the existing concepts of resistance and tolerance strategies (Svensson and Råberg, 2010) that arise in antagonistic co-evolution. In contrast to both resistance and tolerance strategies, resilience does not arise as a defensive strategy in response to deception. Instead, resilience traits are pre-existing in deceived species, rather than evolving as a defence strategy (Vitale and Best, 2019). Resilience, like tolerance, is distinct from resistance in that neither act to lower exploiter fitness and ameliorate the costs of exploitation.

Resilience, however, should always increase exploiter fitness (whereas tolerance does not impact exploiters fitness; Best et al., 2014). Resilience does not exclude the evolution of

adaptations and counteradaptations but may weaken the costs that drive it. Resilience could also operate in concert with one of the other hypotheses for the maintenance of deceptive systems, low encounter rates, but seems unlikely to be compatible with the indirect benefits hypothesis as resilience does not involve any benefit to the exploited species.

A putative resilience trait in the context of sexual deception is haplodiploidy. Sexually deceptive orchids are almost exclusively pollinated by solitary hymenopterans (Gaskett et al., 2008; Kai-Fong et al., 2019). All Hymenoptera (wasps, bees and ants) are haplodiploid and have one ancient, arrhenotokous origin (Normark, 2006). Diploidy has never evolved in the hymenopterans, and the most prevalent form of haplodiploidy in the Hymenoptera is arrhenotokous haplodiploidy, where diploid females arise from fertilised and haploid males from unfertilised eggs (Heimpel and de Boer 2008). Thus, mated females can produce daughters and sons (by controlling the number of fertilised vs. unfertilised eggs) and unmated or sperm depleted females can produce only sons. A few hymenopterans are not arrhenotokous, and instead exhibit thelytoky, where unfertilised females produce only daughters. I note that under arrhenotokous haplodiploidy, diploid males can arise – but they are usually inviable or infertile and so do not contribute to population reproduction (Harpur et al., 2013; Winkert et al., 2019).

Because haplodiploid females can reproduce without sperm (albeit, all their offspring are male; Godfray, 1990) and can facultatively adjust the sex ratio of their offspring when

they do (Burton-Chellew et al., 2008; Booksmythe et al., 2017), female mating failure (through sperm loss or missed mating opportunities due to orchids) could drive an increase in sons from generation to generation, causing a male-biased sex ratio (Figure 2). In turn, this male-biased sex ratio would reduce the costs of deception to the pollinator population, but also provide a benefit to the orchids. There would be new, naïve males for orchids to exploit, but also enough males in the population to keep some females fertilised and producing daughters. In this way, the population might persist over time. This contrasts with a sexually deceptive orchid targeting a diploid pollinator species, where the pollinator cannot reproduce without matings and sperm.

A defining feature of this resilience hypothesis is that resilience traits simultaneously buffer the costs of exploitation and prevent escape from exploitation. By reducing costs, resilience traits reduce the risk of extinction and weaken selection for counteradaptation – preventing escape. This could be intensified for haplodiploidy, as haploid males evolve slower than diploids, with sons arising from unfertilised eggs (Carrière, 2003). As such, any selection on males can only be from grandfathers to grandsons (rather than from fathers to sons as in diploids). This suggests haplodiploids are a perfect target for exploitation: the females can cope with reduced access to sperm or mates, and the males are less likely to evolve discriminatory abilities or other traits to avoid orchids.

Haplodiploidy could be critical for the evolutionary maintenance of sexual deception because it could mitigate the costs of orchid interference in pollinator reproduction.

Indeed, it may be that hymenopterans are often pollinators of sexually deceptive orchids due to haplodiploidy, and diploid-orchid interactions have become extinct, while haplodiploid-orchid interactions have persisted. The preponderance of haplodiploid pollinators for deceptive orchids is particularly striking when comparing the relative proportions of haplodiploids and diploid pollinators across different pollinator strategies (Figure 4). Using the Atlas of Orchid Pollination (Van der Cingel, 2001) for an overview of pollinators and pollination strategies, I found that the proportion of haplodiploid pollinators may relate to pollination strategy. Rewarding orchid species and food deceptive orchid pollinators are approximately equally split between diploids and haplodiploids, whereas sexually deceptive orchid species have mostly haplodiploid pollinators (90%, Figure 4). Orchids with other deceptive pollination systems such as brood site deception and sensory traps are also predominantly pollinated by non-haplodiploid pollinators. I note that the deception of hymenopteran pollinators (an entirely haplodiploid order) appears to be the ancestral state in at least Orchidinae (which includes the large European sexually deceptive genus *Ophrys*; Inda et al., 2012; Figure 3A), and I predict it may be ancestral for the sister tribe Diurideae (which includes all 9 of the Australian hymenopteran-pollinated sexually deceptive genera) which shares the same subfamily Orchidoideae.

Exploring the effect of pollinator type on pollination rates as well as whether pollinator-shifts have taken place from diploid to haploid (and the rate of any shifts) would help to

confirm or deny the hypothesis that haplodiploidy is a resilience trait critical to the success of sexual deception. Intriguingly, *Lepanthes* orchids may provoke ejaculation in their diploid pollinator (Fungus gnats; Blanco and Barboza, 2005). However, fungus gnat sex ratios become more male-biased at cool temperatures (Farsani et al., 2013; Pandey and Tripathi, 2008; Nigro et al., 2007). In colder weather (April – June), at times when *Lepanthes* orchids flower, fungus gnat populations might have a male-biased sex ratio. This mechanism may help with persistence but remains to be tested empirically.

1.7 Resilience: a general mechanism

This concept of resilience could be useful for explaining persistence in other asymmetric co-evolutionary model systems. Male killers such as *Wolbachia* or *Spiroplasma* influence adult sex ratios by decreasing the number of produced sons, which limits the availability of mates for females (Pontieri et al., 2017; Hayashi et al., 2016). These bacteria occur in several hymenopteran species (Gerth et al., 2011; Hoy et al., 2003; Pannebakker et al., 2004; Goerzen and Erlandson, 2018). Perhaps haplodiploidy acts as a resilient trait here too, to enhance the persistence of the exploited species and its exploiter.

Besides haplodiploidy, other resilience traits may be present in and explain the persistence of parasite-host interactions. For instance, individuals that are heterozygous for sickle-cell anaemia are protected against the danger of dying from malaria but act as good vectors through which the parasite can reproduce and spread (Luzzatto, 2012).

Another resilience trait could be parental investment strategies, for instance, K versus r strategists. This may have implications for the persistence of prey: species with longer life expectancies and fewer offspring may be less able to cope with predation than species with reduced investment in individual offspring, but high investment in producing many offspring (Pianka, 1970).

Similarly, resilience may be conveyed via differences in developmental modes of cuckoo hosts (altricial, or early development state at birth, versus precocial, or late development state at birth) may play an important role in understanding the long-term stability of obligate interspecific brood parasitism (Yom-Tov and Geffen, 2006). Three of the four orders of species that exhibit obligate interspecific brood parasitism (Cuculiformes, Piciformes, and Passeriformes) are altricial, while only one order (Anseriformes) is precocial. There is a greater duration of parental care in altricial species that might allow parasitised individuals time to reduce the costs of deception (Sheriff et al., 2018), and yet the appearance of altricial young may allow parasitic chicks to remain inconspicuous for longer (Scheiber et al., 2017). Additionally, because precocial species have longer incubation times and invest more in eggs, the cost of rejecting an egg may be greater for them, whereas an altricial species may reject an egg and re-lay (Augustine et al., 2019), with lower costs.

Determining whether these traits confer resilience would be difficult to track in the real world during a researcher's lifetime. Mathematical modelling, paired with experiments

that test the outcome of these models, offers promise for determining how exploited species cope (Kokko et al., 2008). Modelling could also incorporate and test interactions between resilience and the other three hypotheses for the maintenance of deceptive relationships (low encounter rates, indirect benefits, co-evolution). Furthermore, meta-analyses that utilise existing knowledge and explore the preponderance of putative resilient traits in antagonistic relationships (parasitism or predation, for example) and the impact on exploiters (in terms of mortality rates, infection rates or reproduction rates) might reveal the importance of resilience as a mechanism.

Resilience is an exciting new avenue of research in the field of behavioural and evolutionary ecology that can help answer questions of persistence in exploitative relationships. This mechanism may shape the coexistence of exploitative species and their hosts, and help us understand that which was previously inexplicable.

1.8 Conclusions

- (1) Deception is a common strategy utilised by a variety of species but is extremely common in the Orchidaceae. The origins of sexual deception remain unclear, and much more research is required in this field. It seems likely that sexual deception arose from food-deceptive orchids. If so, this suggests that deception arose, and might be maintained, through co-evolution.
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- (2) How do exploited species persist? The three mechanisms that might explain this are not useful (in the case of low encounter rates and indirect benefits), or do not have an adequate amount of evidence to support the idea (counter-adaptations) in the context of sexual deception. Research that focuses on both plant and pollinator would help confirm or reject the hypothesis that orchids impose selection on their pollinators.
- (3) Orchids are under strong selection from their pollinators, but few studies have attempted to quantify costs or selection that deceptive orchids might impose on their pollinator. Initial studies suggest costs such as missed matings or sperm wastage correlate with behavioural changes such as male and female avoidance of orchids and male strategic ejaculation. Systematic studies would allow a more exact understanding of costs and better evaluate the possibility of reciprocal co-evolution or an arms race.
- (4) I propose a new general mechanism to explain the persistence of exploited species in asymmetric co-evolutionary relationships: 'resilience' traits buffer costs to exploited individuals, but uniquely limit their ability to evolve and escape from exploitation. Resilience is distinct from resistance and tolerance as it does not arise from exploitation but is pre-existing. Moreover, it always confers a fitness benefit to the exploiter.
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- (5) I present a putative resilience trait to test this mechanism: haplodiploidy, in which females deprived of sperm by orchids may still reproduce – although all her offspring would be sons. Approximately 95% of sexually deceived pollinator species share this trait. An overproduction of sons in response to sperm limitation may benefit orchid pollination while providing enough males to keep females fertilised and maintain a population. Moreover, haplodiploidy slows down the rate of trait evolution, and the changes in the sex ratio could have knock-on effects for male behaviour that render them 'ideal' pollinators.
- (6) Resilience may be useful in understanding the persistence of parasitic interactions, as well as predator-prey interactions. I hope that resilience can be broadly other asymmetric co-evolutionary model systems.

1.9 Tables and figures



Figure 1: *Cryptostylis subulata* tricks its ichneumonid pollinator, *Lissopimpla excelsa* into ejaculating and wasting its sperm, while gaining pollination. Sperm loss and missed mating opportunities could impose great costs on deceived populations – how does this relationship persist? Image courtesy of C. Young.

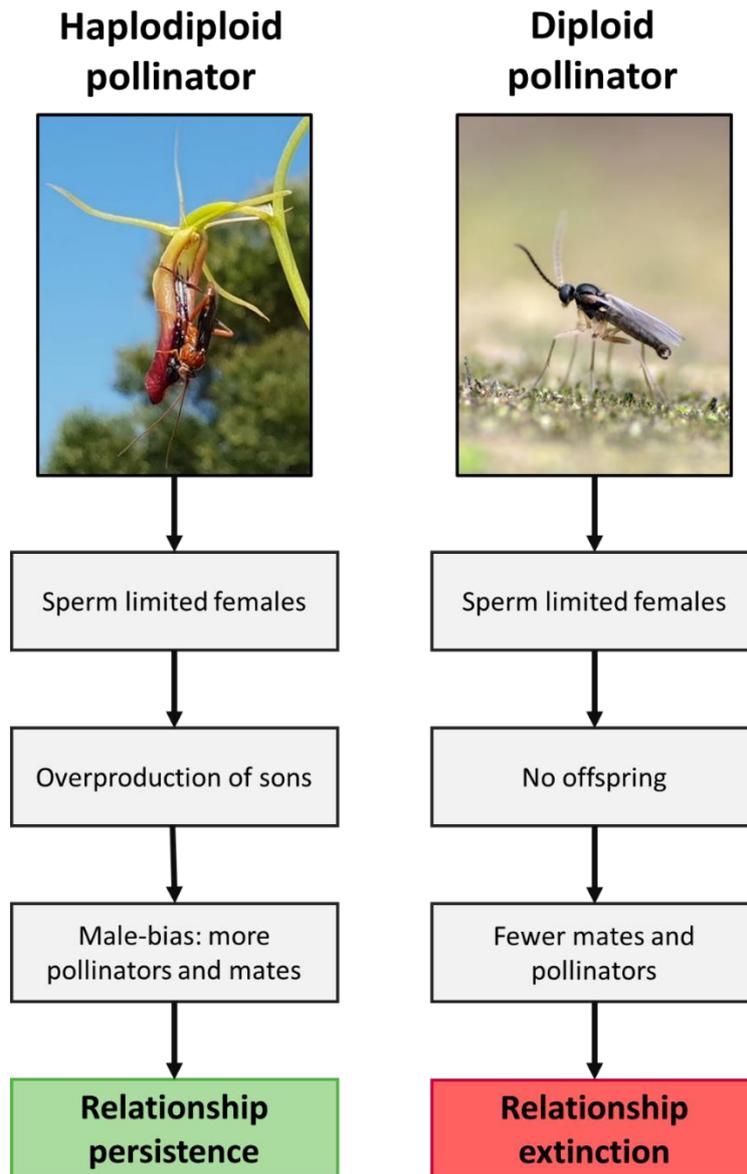


Figure 2: Reproductive strategies of pollinators (haplodiploid versus diploid) and their hypothesised outcomes if sexually deceptive orchids induce sperm or mate limitation in pollinator populations. I present haplodiploidy as a putative resilience trait that permits persistence in the context of sexual deception. This resilience trait buffers the costs that extreme deceivers, such as *Cryptostylis* orchids (*C. subulata*, top left), impose on their pollinators. Images courtesy of C. Young (haplodiploid wasp, *Lissopimpla excelsa*) and K. Schulz (diploid fungus gnat, *Odontosciara nigra*).

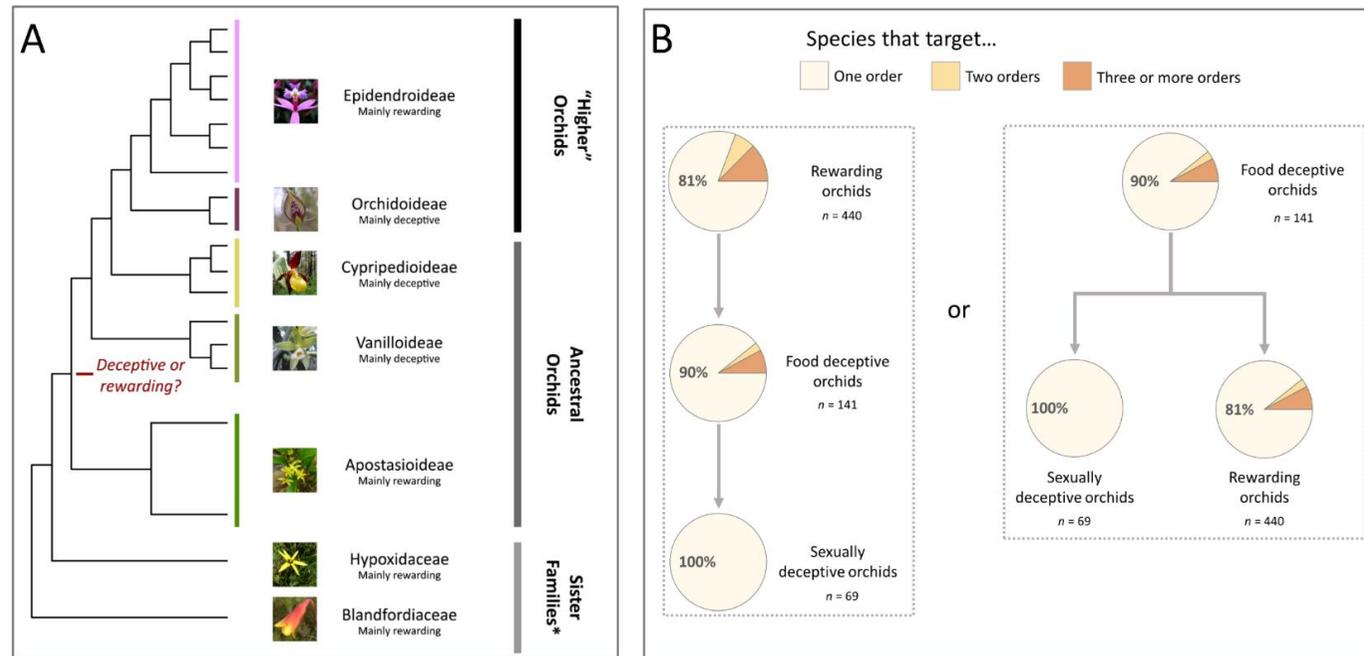


Figure 3: (A) Phylogeny of Orchidaceae with proposed sister families, modified from a molecular analysis by Kocyan et al. (Kocyan et al., 2004; Inda et al., 2012; Cozzolino et al., 2001). I provide examples from each sub-family: *Epidendrum secundum* (Epidendroideae); *Cryptostylis erecta* (Orchidoideae); *Cypripedium calceolus* (Cyripedioideae); *Vanilla chamissonis* (Vanilloideae); *Apostasia wallichii* (Apostasioideae); *Spiloxene capensis* (Hypoxidaceae); and *Blandfordia grandiflora* (Blandfordiaceae). Images sourced from creative commons. (B) Overview of pollinator types (data from Van der Cingel, 2001) to demonstrate the evolution of Orchidaceae from generalist to specialist or specialist to generalist proposed by various phylogenies; specialist/generalist indicated by the proportion of species in each category that have pollinators of one order, or pollinators from several orders. Pollinator type (targeting one order, two orders or three or more orders) appears to relate to pollination strategy.

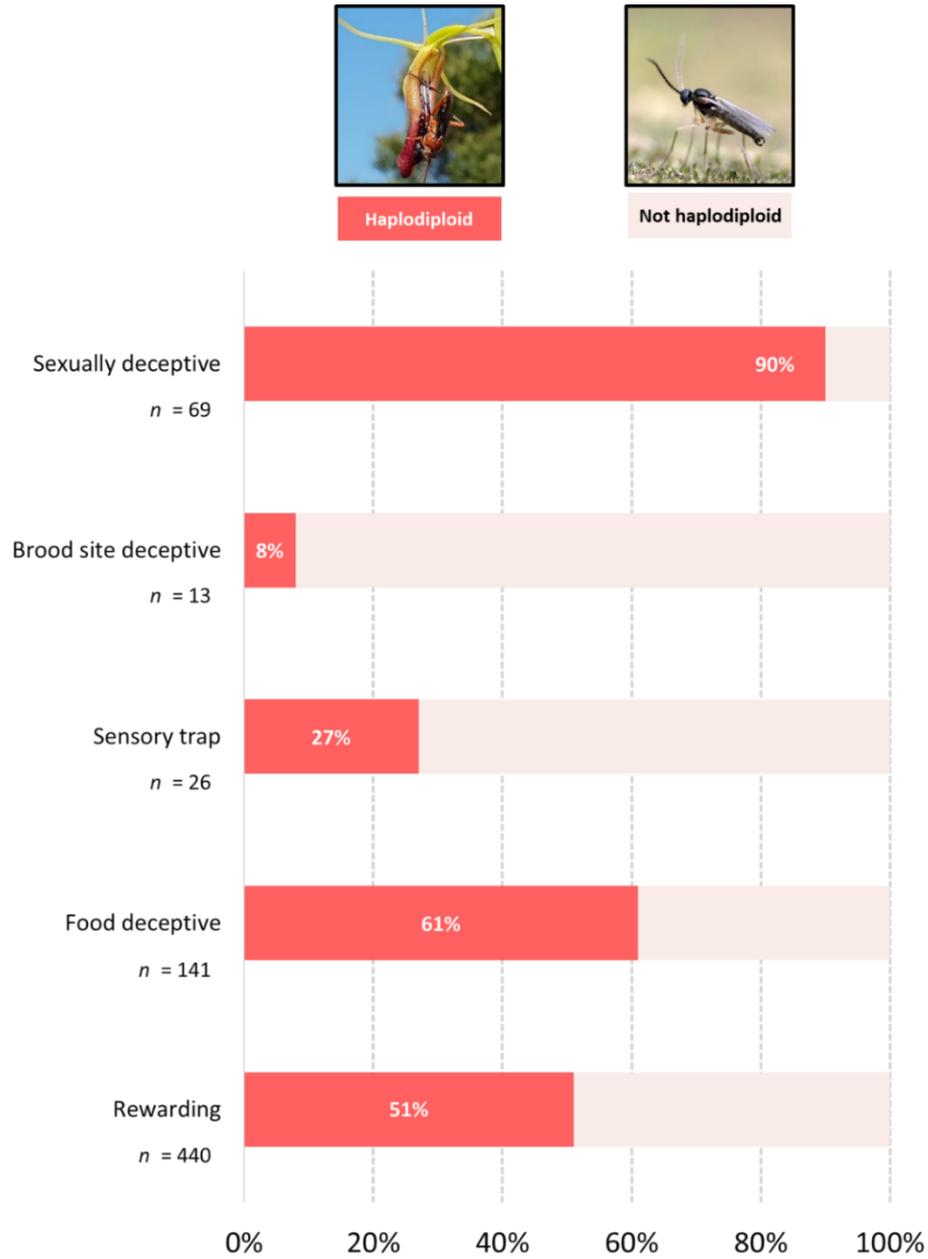


Figure 4: Relative proportions of haplodiploid and not haplodiploid pollinators across different known pollination strategies for 755 species of Orchidaceae (excluding autogamous orchids; data from Van der Cingel, 2001). Pollinators included Hymenoptera, Diptera, Coleoptera, Lepidoptera and birds. Pollinator type (haplodiploid or not) appears to relate to pollination strategy. Pollination strategies that were not rewarding, but otherwise unclear were described as 'sensory traps'.

Table 1: Description of the main types of deception exhibited in orchids and hypothesised mechanisms for the maintenance of those relationships. "?" indicates areas where more research is needed.

Deception	Number of genera	Pollinator	Exploited behaviour	Cost to pollinator	Persistence mechanism	Reference
Food deception (Batesian and Generalised)	47	Social and solitary bees, butterflies and flies.	Foraging	Foraging time	Low encounter rates (?) and learned avoidance, achieved as mimic occurs in sympatry with models (Batesian) and is rare, or many Polymorphisms (Generalised).	Internicola and Harder, 2012; Schiestl and Johnson, 2013; Jersáková et al., 2009
Sexual deception	18	Mostly Hymenoptera ; some Diptera	Reproductive	Time taken to locate genuine females; Sperm wastage.	Low encounter rates (?); Learned avoidance (?)	Gaskett et al., 2008; Wong and Schiestl, 2002; Cozzolino and Widmer, 2005; Vereecken, 2009; Gaskett, 2011
Brood site deception	11	Diptera and Coleoptera, some Hymenoptera	Reproductive	Waste time for finding true breeding sites; deposit and lose eggs on mimic brood site	Low encounter rates (?); High fecundity of targeted spp. (?)	Stöckl et al., 2011; Urru et al., 2011; Roy and Raguso, 1997

Chapter 2: Resilience of pollinators against orchid sexual deceit.

In review in Nature

2.1 Introduction

Deceptive organisms manipulate other species with sensory stimuli (Mokkonen and Lindstedt 2016). Assassin bugs imitate insect vibrations to lure spiders (Wignall and Taylor 2011); praying mantises resemble flowers to attract insect prey (O’Hanlon, Holwell and Herberstein 2014); cuckoos mimic host eggs (Yang, Liang and Møller 2017); and sexually deceptive orchids fool male insects into mating with and pollinating flowers (Gaskett, Winnick and Herberstein 2008). The coexistence of deceptive species and their ‘victims’ is an intriguing eco-evolutionary problem, given that deception imposes acute fitness consequences (including death or reproductive loss) on the latter.

Asymmetric relationships can be maintained via co-evolutionary arms races, as for cuckoos and their hosts^{7,8}. Alternatively, costs to duped species may be mitigated by low encounter rates (Flower, Gribble and Ridley 2014; Kokko and Rankin 2006; Kokko, Heubel and Rankin 2008), or indirect fitness benefits, as when sexually

cannibalised males gain improved paternity (Barry, Holwell and Herberstein 2008; Berning et al., 2012; Welke and Schneider 2012; Wu, Zhang, He, Liu and Peng 2013). Sexual deception is a surprisingly common pollination strategy, especially in orchids, as it comes with no evidence for counter-adaptations, low encounter rates or indirect benefits. It occurs in ~400 spp. worldwide (Gaskett 2011), originating at least 18 times independently, mostly in Australasia and the Americas, but also Africa and Europe (Cozzolino and Widmer 2005; Gaskett 2011; Jersáková, Johnson and Kindlmann 2006). Sexual deception is generally considered a benign coevolutionary interaction: although the flowers evolve to exploit pollinator preferences (de Jager and Peakall 2019; Phillips and Peakall 2018), it exerts negligible impacts on pollinator fitness.

I challenge the above interpretation and show that sexual deception may impose severe costs, with evolutionary consequences. *Cryptostylis* orchids trigger ejaculation and sperm wastage by their single shared pollinator (Gaskett, 2012), males of the solitary parasitoid wasp *Lissopimpla excelsa*, (Ichneumonidae; Pimplinae; Gaskett et al., 2008). Sperm wastage could be costly for solitary parasitoid wasps, exhausting sperm supply (Boivin, Jacob and Damiens 2005; Damiens and Boivin 2006; Jager and Ellis 2014; Olsson, Madsen and Shine, 1997), and in *L. excelsa* males likely become at least temporarily sperm-depleted (Brunton Martin et al., 2020; chapter 4). Sexually deceived males typically fail to find real females among false orchid signals (Wong,

Salzmann and Schiestl 2004; Jager and Ellis 2014; Wong and Schiestl 2002), raising the question of pollinator persistence when falling victim to exploitation by orchids. To date, there is no evidence that sexual deception drives counter-adaptations. Low encounter rates are unlikely for *Cryptostylis*, as evidenced through very high pollination success (up to 90% vs. 30% global mean for other sexually deceptive orchids; Gaskett 2011) and occurs in high density patches (up to 900 plants; Gaskett and Herberstein, 2006). The possibility that these patches exist through exploiting naïve individuals that migrate into the population from surrounding areas is unlikely, due to the short dispersal distance of *L. excelsa* (minimum: 16m; maximum: 625m; mean: 99m (Weinstein, Davis, Menz, Dixon and Phillips, 2016). Finally, sexual deception provides no conceivable indirect benefits to pollinators.

I study here the potential ‘resilience’ traits of the pollinator that aid its persistence. Any life-history trait that protects an exploited species from the costs imposed by an exploiter can be considered ‘resilience’, and it comes with a flipside of potentially weakening selection or counter-adaptations against exploitation. Intriguingly, 95% of sexually deceptive orchids are pollinated by haplodiploid species (solitary bees/wasps), whereas rewarding and food deceptive orchids have a wider and more typical range of pollinators (social bees/wasps, flies, butterflies, moths, beetles; Cingel, 2001; Gaskett, 2011). I consider haplodiploidy a potential resilience trait, since females (if fertilised) can facultatively adjust their offspring sex ratio by

controlling the proportion of their eggs that are fertilised (Burton-Chellew et al., 2008), and can reproduce when deprived of sperm (although all her offspring will be male). In other words, a population of mothers of haplodiploid species comprise virgin male-producers and mated mothers who specialise in daughter production (to an extent, as they may also produce some inviable diploid males; Heimpel and de Boer, 2008). These features may make individuals more resilient when exploited by orchids. If sperm limitation automatically increases male production, the next generation will have improved male availability. In turn, this should provide enough males to fertilise females and help maintain the population despite some male mating effort being wasted on orchids. In this way, sexually deceptive orchids with a haplodiploid rather than diploid pollinator may have a double advantage: a pollinator that can still reproduce despite orchid interference and an artificial upsurge in male insects to share sperm between females and orchids. The exploited pollinator neither goes extinct, nor escapes from orchid exploitation.

Here I examine the above logic with a combination of mathematical modelling, and field experiments to test if sexual deception drives differences in pollinator populations in a manner that contributes additional elements to resilience. In field experiments, I compare morphological and behavioural traits of wasps that do and do not live in sympatry with orchids. I hypothesise that sexual deception causes changes that might act as counter-adaptations. Traits that mitigate the costs of deceit or

help avoid deceit include longer antennae or larger eyes, facilitating the ability of males to discriminate between an orchid and a real female. Likewise, larger testes or larger sperm stock could help males cope with the costs of ejaculation on orchids. My model tests whether haplodiploidy acts as a resilience trait, buffering the costs of sexual deception. I compare the predictions of the model to a survey of museum and digital collections.

2.2 Are there morphological or behavioural responses to sexual deceit?

I tested for differences in morphology or behaviour at three sites with and three without natural populations of orchids. I recorded males' (males at sites with $n = 60$; sites without $n=59$) behaviour before, during, and after copulation with an experimentally presented orchid bait. Once captured, I measured their morphological and sensory structures related to detection (e.g. antennae, eyes) or sperm capacity (e.g. testes, seminal vesicles) and assessed sperm stock in testes from males caught before or after mating as well as that in spermatophores left on orchids.

Exploratory principal component analysis and MANOVA revealed a significant difference between male traits from sites with orchids versus males from sites without (p value < 0.01 , S. Table 1). The only apparent morphological difference of those

tested (see Methods) is that males from sites with orchids have longer antennae relative to their body length (mean at sites without: 0.98 mm/mm body length \pm 0.07 s.d. ; sites with: 1.1 \pm 0.05 s.d. ; Fig. 1 A). In terms of behaviour, at sites with orchids the time taken for the first male to arrive at the bait was longer (mean at sites without: 150s \pm 102 s.d. ; sites with: 341s \pm 243 s.d.; Fig. 1 A Fig. 1 B), males spent less time mating with orchid bait (mean at sites without: 41.6s \pm 8.2 s.d. ; sites with: 21.3s \pm 11.3 s.d.; Fig. 1 D) and, when males ejaculate, they deposit less sperm than males at sites without orchids (mean at sites without: 252 spermatazoa / mm body length \pm 120 s.d. ; sites with: 133 spermatazoa / mm body length \pm 80.7 s.d.; Fig. 1 F). At these same sites, males were less likely to pollinate an orchid bait, but just as likely to ejaculate on orchid bait as males from sites without orchids (Fig. 2 A and B).

2.3 Does antennae length improve discrimination in sexually deceived wasps?

I predicted that longer antennae would correlate with increased discriminatory behaviour if it is a counter-adaptation to deception. Increased antennae length did not appear to confer discriminatory ability. At sites with orchids, faster times for first male arrival were associated with longer antennae (interaction GLM p value $<$ 0.01) but males mated for a shorter amount of time (interaction GLM p value $<$

0.01). These same relationships were not apparent at sites without orchids (Fig. 1 C and E). Longer antennae did not significantly correlate with ejaculate size (Fig. 1 G, p values > 0.05). Notably, the number of failed trials (where no males came into an orchid bait) did not differ between sites with and without orchids (S. Table 2)

2.4 Does haplodiploidy help build resilience?

When sexual deception elicits sperm wastage, the costs of deception might be reduced for haplodiploid pollinators compared to diploid pollinators. My model tests the idea that haplodiploidy confers resilience to deception, incorporating factors known to influence ichneumonid wasps' local population dynamics (particularly male and female reproductive traits) and varies key traits to estimate the effect of orchids on hypothetical diploid and haplodiploid populations (see Methods for details).

I base the haplodiploid version of this model on the life cycle of ichneumonid wasps, with males emerging before monandrous females (protandry; Quicke, 2014). Females stop investing in pheromone production after mating, irrespective of whether they have received a sufficient amount of sperm (Ridley, 1993). Under ideal laboratory conditions, female ichneumonids typically produce between one and four eggs daily. As the rate of egg production is phylogenetically constrained across parasitoid groups (Jervis, Moe and Heimpel, 2012; Price, 2003), I use these estimates in the model. Egg production lasts throughout a female's lifespan, and most females will begin

oviposition after emerging, regardless of whether they have mated (Ode, Antolin and Strand, 1997).

Males have a finite capacity, defined as the number of times each male can mate before being classified as sperm depleted. Sperm depleted males will continue to mate, but will not transfer any sperm to females (Boivin et al., 2005). Monandrous females mating with such males become ‘pseudo-virgin’, constrained to produce only sons for life. I assume that the populations coexist with orchids long enough for sex ratio adjustments to evolve; thus the sex ratio produced by mated females is allowed to depend on the son production by virgins and pseudo-virgins (Rautiala, Helanterä and Puurtinen, 2017; since I assume this is an evolutionary response, I do not assume that females directly measure son production of others, but that females producing optimal sex ratios have been selected for).

The diploid version of the model uses the same life cycle, except for the consequences of diploidy: unmated females, or females who mated with sperm-depleted males, cannot produce offspring of either sex, and those with sperm produce offspring with a 1:1 sex ratio.

I found that haplodiploid populations were able to persist where diploids do not.

Every value male capacity could take (e.g. 1 ... 30) associates with a threshold density of orchids, above which the pollinator population becomes extinct (Fig. 3),

and those thresholds are higher for haplodiploid populations. Unsurprisingly, as male capacity increases, populations can tolerate higher densities of orchids (all curves increase in S. Fig. 1). The persistence threshold is always higher for haplodiploids than for diploids, supporting my hypothesis that haplodiploidy buffers against the negative impacts of exploitation by orchids and thus enhances the stability of the two-species system. In S. Fig. 1, the difference between haplodiploids and diploids arises across all examined values of male search rate (s), female emergence (T_P) or egg production (f).

Both in diploids and haplodiploids, population persistence in the presence of orchids is strongly impacted by the efficiency of male searching and the day females emerge (S. Fig. 1). High male search efficiency will lower population persistence (contrast $s = 10$ with $s = 1$ in S. Fig. 1). If I totally remove protandry (i.e. males and females emerge and mate on the same day), both haplodiploids and diploids can persist up to extremely high orchid densities (S. Fig. 2).

I tested whether the predicted shift in sex ratio was observable in nature by comparing the number of male and female museum specimens of the pollinator of *Cryptostylis* orchids, *L. excelsa*, collected from sites with and without orchids (n = 256 specimens). I also included male and female specimens of a sister ichneumonid *Echthromorpha intricatoria*, that does not pollinate any sexually deceptive orchids that co-occurs with *L. excelsa* and has a similar life history (n = 129). An issue with

museum specimens of orchid pollinators is that male insects may be overrepresented in collections because orchid researchers lodged them. This is because density of records can depend on human interaction and lodging what they find “interesting” (ElQadi et al., 2017). Orchids are particularly interesting to humans – and most species are classified as endangered due to over-collecting (Teoh, 2019). Hence, a male wasp on an orchid might be easier to find and lodge than a female probing in grass. To avoid this and other biases, I carefully and systematically examined every record and excluded several (see Methods). A generalised linear model reveals that *L. excelsa* is significantly more male-biased at sites with orchids than at sites without orchids (p value 0.004; Fig. 4). Overall, collections from New Zealand were more male biased than from Australia (p value 0.004; Australia: 40% male ; New Zealand: 61% male). This is consistent with my model’s prediction that when orchids trigger pollinator sperm wastage on flowers, this can boost the production of sons from sperm-deprived female insects, resulting in a male-biased pollinator population.

2.5 Discussion

Males that coexist with sexually deceptive orchids have different behaviour and morphology to those that do not.

I found evidence that suggests sexual deceit by orchids selects for changes in pollinator morphology and behaviour.

Sexually deceptive orchids attract their pollinators primarily by mimicking female insect sex pheromones (Gaskett and Herberstein, 2009), which insects, including Ichneumonids, typically receive via chemoreceptors on their antennae (Chapman, 1982; Grosch, 1947). *L. excelsa* will readily copulate with a simple pin that does not resemble a female insect, as long as it is scented with *Cryptostylis* flower extract (Bohman, Weinstein, Phillips, Peakall and Flematti, 2019). I found that pollinators from sites with orchids had longer antennae relative to their body size (mean difference 0.1mm/mm body length; MANOVA p -value < 0.001 ; ANOVA F - value: 39.8), indicating an increased investment in sensory structures compared to pollinators from sites without orchids. In insects, larger antennae are typically associated with more olfactory receptors, with more receptors allowing greater sensitivity to chemical signals (Chapman, 1982).

At orchid sites, it also took longer for first male arrival to an orchid bait.

Additionally, males were less likely to mate with an orchid bait, and when they did mate, they had shorter mating durations. These behavioural changes may be adaptive responses to orchid deception, but they do not correlate to behaviour in a way that I would expect if longer antennae improve discrimination. Instead, antennae length correlated with shorter times for first male arrival, shorter mating durations, and an increased likelihood to pollinate the orchid. At both sites with and without orchids, males were just as likely to ejaculate on the exposed orchids.

Rather than being traits that have arisen from an arms race, longer antennae can arise as a conceivable consequence of sexually deceptive orchids enhancing scramble competition – a behaviour observed in most solitary, parasitoid wasps (Quicke, 2014). As demonstrated in this model, sexual deceit eliciting sperm wastage in haplodiploids increases the number of males in a population. With more males to compete with, selection may act to produce males better suited for scrambling: longer antennae for increased detection, and small body size for increased mobility (Herberstein, Painting and Holwell, 2017). Furthermore, shorter times until first male arrival correlated with longer male antennae, possibly due to more efficient searching (but not necessarily discrimination or reluctance to mate). First male arrival time to a bait was, overall, longer at sites with orchids. Because I could not follow a focal male in this study, it is uncertain to what extent the arrival times reflect variations in male searching efficiency or, instead, spatial variation in population density of males, females and orchids impacting how quickly one orchid bait is found. If adaptation is occurring, it may be that a trait other than antennae length is responsible for modulating behaviour.

Scramble competition also predicts changes in mating behaviour and sperm allocation. Here, I observed shorter mating durations and smaller ejaculates at sites with orchids. When females are monandrous, but a male-biased sex ratio is present, sperm competition is expected to be low, while there is heightened competition for

location of females (Parker and Pizzari, 2010). Consequently, these males should invest in obtaining a high number of mates, at the cost of smaller ejaculates (Parker and Pizzari, 2010). Males may also vary ejaculate allocation, or not mate in response to current cues if they become aware that their mate is an orchid, or perceive the orchid as a low-quality female (Ferdy, Gouyon, Moret and Godelle, 1998; Gaskett, 2011). The males may also be sperm depleted (or close to sperm depletion): in this study, I observed three wasps with less than 1000 sperm in their seminal vesicles (who did not leave ejaculate) – indicating they were sperm depleted. When males become sperm depleted, they will continue to mate: transferring very little sperm, or only seminal fluid without sperm (Boivin et al., 2005).

Finally, I note that males analysed here are those who responded to the presentation of an orchid, and so these results may exclude any males that never respond to orchids. Capturing an unbiased sample of the populations would verify whether these differences arise from scramble competition or counter-adaptations. It is also important to note that detected differences may also arise due to differences in site. While I endeavoured to make sites with and without orchids as comparable as possible (S. Table. 4), they are not precisely matched – particularly in terms of vegetation structure. This means that the driving force for differences in behaviour between these populations may be differences in habitat. For instance, if hosts are

limiting at one site, we would expect males to disperse more (Perrin and Mazalov, 2000) and search more actively for mates than at sites where host aren't limited.

Further, differences in habitats might support larger or smaller populations of wasps. If male populations are not precisely matched, the differences observed here might be attributable to differences in rates of encounter. Given that sticky traps did not adequately assess pollinator abundance, one way to estimate male encounters would be through a mark-recapture study as per the methods in Weinstein et al., 2016.

Long term impacts of sperm wastage on haplodiploid and diploid populations.

If a substantial proportion of the male population are sperm depleted, males and females may experience reduced fitness. My model shows that the stability *Cryptostylis* orchids and their haplodiploid pollinators may thus be partly explained by sperm depletion causing more pollinators to be created (via male production of unmated females). Although my model simply derived consequences of specific, fixed, orchid densities for the pollinator and thus did not track pollination success or the consequences for orchid dynamics, the results suggest it may be advantageous for orchids to mimic females sufficiently well to elicit ejaculation (rather than pseudo-copulation).

Should this lead to unmated females in the local pollinator pool, there will be more male pollinators in the next generation, and since *Cryptostylis* is a perennial plant, the plant may enjoy elevated pollinating success during its own lifespan.

Haplodiploidy also helps should an orchid subsequently reach densities high enough to cause damage to its pollinator's population dynamics. At a density of orchids where diploids go extinct, haplodiploids can persist with a stable, male-biased sex ratio. This prediction was confirmed using my survey of museum and digital collections, although sampling the at-birth sex ratio in wild populations would be ideal.

Perhaps counter-intuitively, this model suggests that high male search efficiency lowers population persistence. This becomes explicable because high search rates combined with protandry causes there to be some time when searching males only encounter orchids, as females have not yet emerged. Highly efficient males find many orchids to mate with and are already depleted when females begin emerging, particularly so if protandry is strong (females emerge several days after males begin searching for them; in this case, high search by males makes persistence very difficult, (S. Fig. 1 D and H). Despite this, haplodiploid males with high search rates had better production than diploids (compare S. Fig. 1 B and F; D and H). As per my field results, if orchids do create males that are subject to increased scramble competition and search more for females, haplodiploids may well be better able to

cope with this effect as well. Future models might also explore the effect of search rate and male-biased populations on orchid pollination and reproduction success.

The success of *Cryptostylis* may be explained not by the prevention of extinction, but by its creation of more pollinators through ‘sperm theft’. At high numbers of orchids, where diploids go extinct, haplodiploids persist with a stable, male-biased sex ratio.

Haplodiploid females are not only able to reproduce without access to sperm, but are also able to control the proportion of eggs that are fertilized when they do have sperm: thus, allowing them to respond to and stabilize their population’s sex ratio (Burton-Chellew et al., 2008). It could, then, be advantageous for orchids to elicit

ejaculation, especially if this results in not only more male pollinators, but

heightened impacts of scramble competition: naïve males who are also pre-disposed to mating quickly and visiting as many ‘females’ as possible. I re-iterate here, that while I found morphological change in response to orchid deception, it was a small difference and did not seem to correlate with differences in discriminatory behaviour.

Further sampling of wild populations, their sex ratio and counter-adaptive traits, could enhance our understanding of the interaction between sexual deception and haplodiploidy.

Additionally, haplodiploid traits evolve slower than diploids (Carrière, 2003), and selection on male traits is weaker when they rarely encounter females (Kokko and Mappes, 2007) – so *Cryptostylis* may not only be making more pollinators for

themselves by targeting haplodiploids, but also slowing down the rate at which their pollinators acquire a means to escape. I might then expect that there is stronger selection for non-haplodiploid species that are victims of sexual deceit when costs are high. For instance, the fungus gnat pollinators of *Lepanthes* orchids may deposit spermatophores when they are deceived (Blanco and Barboza, 2005) – could they have counter-adaptations in response to sperm loss?

This study adds to the increasing understanding that individuals can have great effects on whole population dynamics, particularly the role that exploiters can have on populations they manipulate. I encourage systematic studies of this system, and others, that might reveal these impacts further. In other systems, I may see similar suites of traits that make the duped ideal candidates for deception, or even exploitation generally. Male-killing bacteria such as *Wolbachia* or *Arsenophonus* occur in several haplodiploid, hymenopteran species (Gerth, Geibler and Bleidorn, 2011; Goerzen and Erlandson, 2018; Hoy, Jeyaprasath, Alvarez and Allsopp, 2003; Pannebakker, Pijnacker, Zwaan and Beukeboom, 2004). These symbionts can influence the number of males born, and therefore may limit the mates (and sperm) available to females. Perhaps in this system, haplodiploidy acts in a similar way as with populations deceived by *Cryptostylis*: more sperm depleted females results in more males, and enough males in the population to cope with limited mates.

2.6 Summary: The ‘resilient’ dupe

What explains the success of a relationship in which one species exploits and imposes great costs on another? Here, I present evidence that suggests *resilience traits*, like haplodiploidy, could help maintain the coexistence of a deceitful signaller and their victims. While behavioural or morphological counter-adaptations may arise in response to exploitation, certain species may be more sustainable hosts because they are resilient to exploitation.

2.7 Tables and Figures

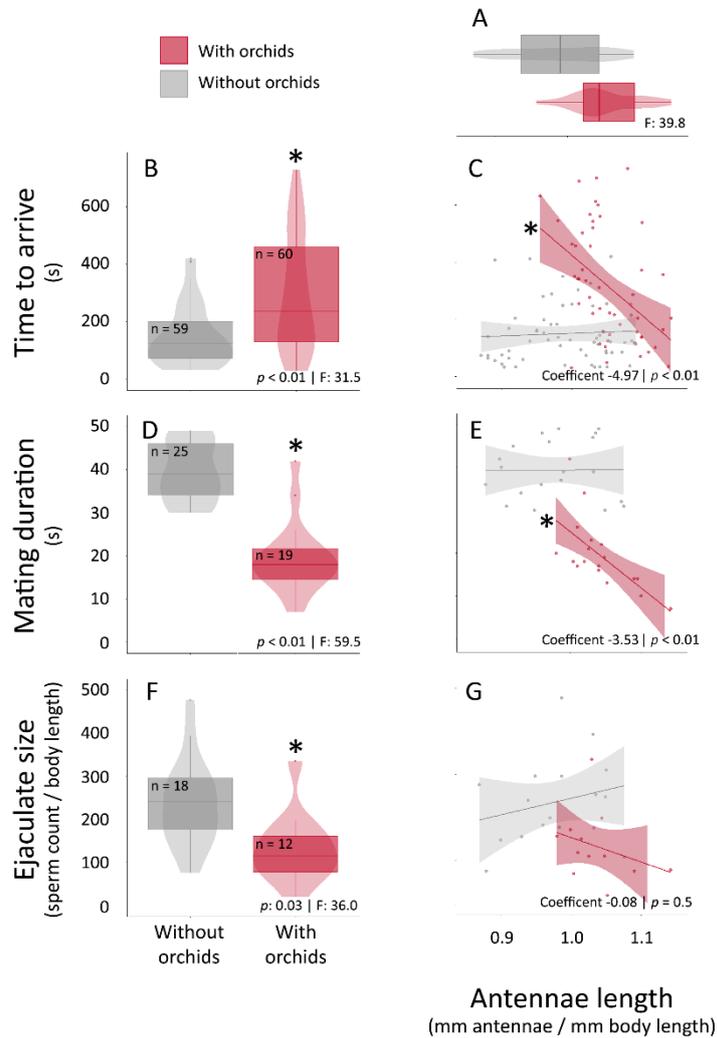


Fig. 1: (A, B, D, F) Principal components analysis revealed that males at sites with orchids, had longer antennae relative to their body length, took longer to arrive at orchids, spent less time mating with orchids and, when they ejaculated they deposited less sperm. (C, E, G) At sites with orchids, longer antennae was associated with a shorter first male arrival time and males with longer antennae had shorter mating durations. Antennae length did not impact the size of males' ejaculates. Reported p values are from results from generalised linear model testing the relationship between antennae length (A), orchid presence and variable (time until first male arrival (B&C): Cohen's f^2 effect size = 0.62, mating duration (D&E): Cohen's f^2 effect size = 3.08 and ejaculate size (F&G): Cohen's f^2 effect size = 0.39). I display untransformed counts, while models used logged values to meet assumptions of normality. Points are raw data, centre bars of box indicate median and inference bands above and

below centre band show the 95% confidence interval. Whiskers mark the range of the data. Sample sizes are displayed on boxes, and smoothed density curve behind.

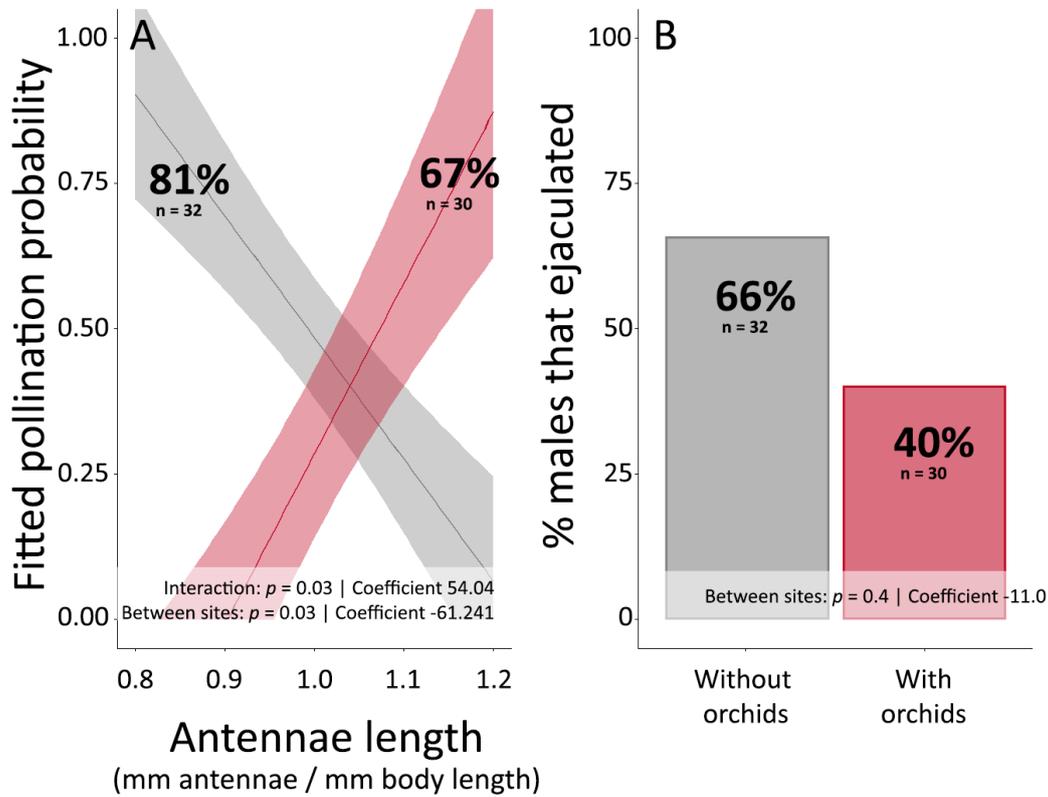


Fig. 2 (A) The probability (line indicates model fit \pm standard error) of males pollinating an experimentally presented orchid bait was smaller at sites with orchids than sites without orchids ($p = 0.03$), but at sites with orchids males with longer antennae were more likely to pollinate an orchid; and at sites without orchids males with longer antennae were less likely to pollinate ($p = 0.03$); Cohen's f^2 effect size 0.203 (B) Antennae length did not impact whether males ejaculated on orchids ($p = 0.5$), and males were just as likely to ejaculate at sites with and without orchids ($p = 0.4$). Note that percentages given on graphs are the raw percent of males that exhibited that behaviour, with sample size; Cohen's f^2 effect size 0.07.

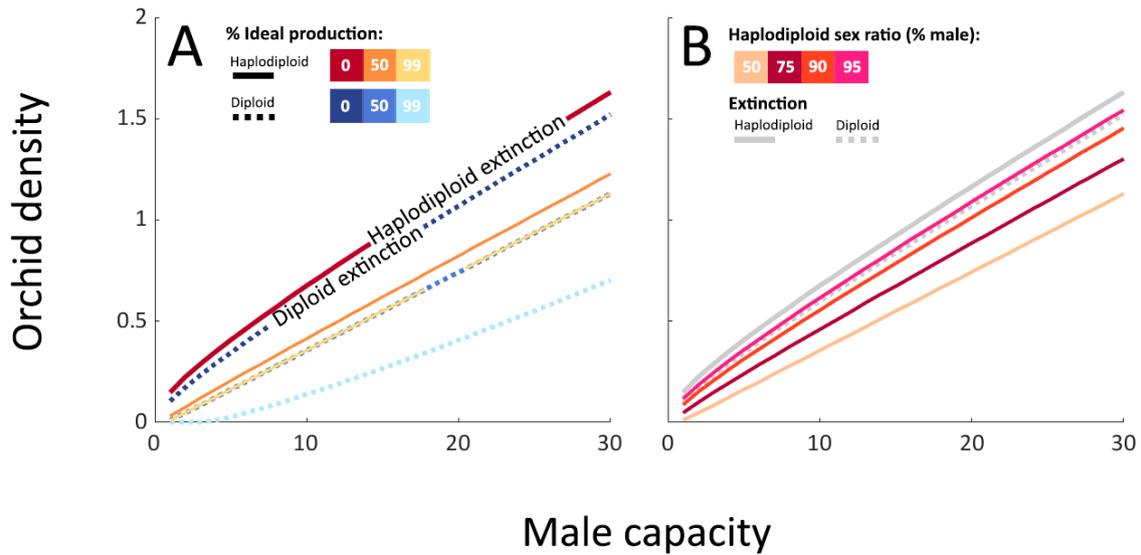


Fig. 3 (A) Daughter production of haplodiploid and diploid populations. Orchid density (D) reflects the number of orchids in the environment per true females, male capacity (c) is the number of times a male can mate before he becomes sperm depleted. Curves indicate the threshold orchid density that permits 0, 50% or 99% of the production level of the best-case scenario to be maintained, with best-case computed within each panel as daughters produced per female when $c = 30$ and $D = 0$. The line reflecting haplodiploid production at 99% is cut away to reveal diploid production at 50%.

(B) Sex ratio predictions. Diploid populations do not adjust sex ratios as there is no production of males caused by matelessness; for these, only the location of the persistence threshold is given, sex ratio is 1:1 everywhere below the persistence threshold, and the population is extinct above it. For haplodiploids, sex ratios are 1:1 underneath the pale orange curve, below 75% male underneath the burgundy curve, below 90% underneath the red curve, and below 95% underneath the purple curve; extinction (via all offspring becoming male) is given in grey.

$f = 1$; $TP = 5$; $s = 5$.

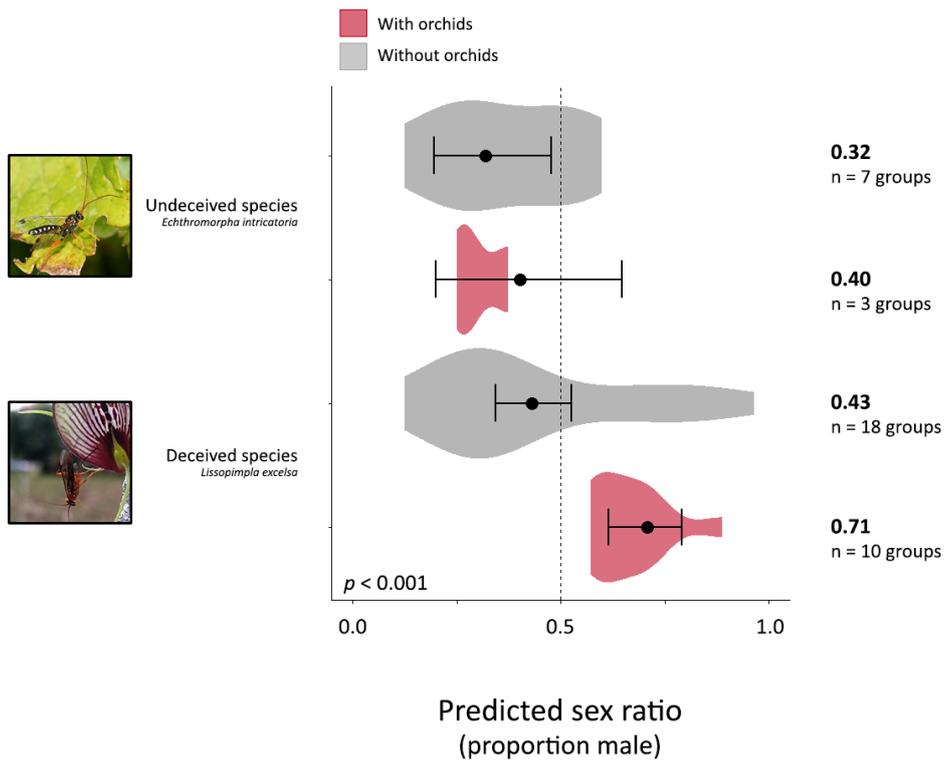


Fig. 4 Predicted sex ratios (text right, and centre points) from binomial generalised model for two species of parasitoid wasps from populations with (red) or without (grey) sexually deceptive orchids. These wasps are either deceived by sexually deceptive orchids (*Lissopimpla excelsa*) or not (*Echthromorpha intricatoria*). Distribution curves are the sex ratios from raw data, error bars indicate a 95% confidence interval. Cohen's f^2 effect size 0.40. Dashed line indicates 0.5 sex ratio (equal investment). Images credit to R. Richter (*E. intricatoria*) and A. L. Brunton Martin (*L. excelsa*).

2.8 Methods

Question 1: Does coexistence with orchids predict morphological or behavioural differences between males?

Site selection

Between December and February 2016-2018, I tested for differences in morphology and behaviour of male *L. excelsa* that are either frequently deceived, or never deceived, by comparing males from three sites with (n = 60 males) and three sites without (n = 59 males) natural populations of orchids in Sydney, Australia (S. Fig 3). I used museum and herbarium (Australian Museum, CSIRO, ANIC, Atlas of Living Australia) records to classify these sites as locations with wasps present and orchids either absent or present. Sites were matched based on the number of orchids present, the number of pollinators available and the number of hosts (*Helicoverpa* observations) for the pollinators available (S. Table 3). In addition, sites were matched based on soil type (information collated from NationalMap, 2020; S. Table 3).

These classifications were confirmed prior to the field season in September 2016, where several 10m transects were used to thoroughly explore for orchids. Sites were at least 2km apart as this exceeds the dispersal range of *L. excelsa* (600m ; Weinstein et al., 2016), and were all sports fields with either planted or native bush present,

and stands of eucalyptus. Unfortunately, as *Cryptostylis* orchids are so abundant in national parks (S. Fig. 3), I was unable to locate sites without orchids contiguous to national parks that also were over 2km from the nearest site or from an orchid patch.

Throughout the course of the study, data on pollination rate (pollen removed), numbers of plants, numbers of orchids and numbers of pollinators (assessed via yellow sticky-traps) were obtained to ensure sites were closely matched.

Field trials

Following the methods of Bower and Brown (1997) I experimentally presented an unpollinated flower of either *C. subulata* or *C. erecta* in the field, in a cage to prevent more than one male visiting at a time. This experiment was also carried out for another study (Brunton Martin, Gaskett and O’Hanlon, 2020) that estimated sperm availability in populations, as such field trials had been randomly assigned to a ‘mating permitted’ (n = 30 at sites with; n = 32 at sites without) or ‘mating prevented’ (n = 30 at sites with; n = 27 at sites without) treatment before commencement.

If no pollinators were attracted after 2 trials, the orchid was removed, and I began a new trial 5 metres away. Orchid flowers were used within 48 hours of collection and, where possible, flowers used in the ‘mating prevented’ treatment were re-used in later

trials. After behaviour was recorded, wasps were caught and killed by freezing within 4 hours.

Behaviour

During ‘mating permitted’ trials, pollinator behaviour was recorded using a voice recorder and video recorder. Behaviours recorded related to either (1) ease of deception: time til first male arrival at orchid bait and whether the wasp landed on the orchid bait or flew past (2) mating effort: copulation duration (associated with sperm transfer ; Barbosa, 2011), total interaction time (time spent on orchid copulating and carrying out post-copulatory behaviour), whether sperm was deposited, or whether pre- or post-copulatory behaviours (wing fanning, antennal grooming, abdominal grooming) were exhibited.

Morphology

All males from ‘mating prevented’ and ‘mating permitted treatments were caught and killed by freezing. Morphology related to either (1) detection: eye area, antennal length; (2) sperm availability: testes area, seminal vesical area; or (3) animal size: abdomen length, body length and abdomen area was photographed with a microscope and measured using ImageJ. Because morphology varies with animal size, all measurements were divided by body length for multivariate analysis.

Sperm stock

Sperm stock was assessed following methods by Bressac et al.(2008): I dissected males seminal vesicles and placed them on a slide with saline (128.3nM NaCl*137mM in phosphate buffer pH 7.2) and 0.3% triton solution to encourage sperm to separate. I broke the vesicles open with an entomological pin and stirred to disperse the sperm. The slide was then fixed with ethanol and left to dry at ambient temperature. Once fixed, I stained the slides for 15 minutes with DAPI (300nM in PBS). Using fluorescence microscopy, I estimated the number of spermatozoa by counting precisely 10 spermatozoa, estimating the area of those 10 sperm, and measuring the area of the sperm mass to obtain the total number of cells in the mass. As sperm number varies with animal size (Bangham, Chapman and Partridge, 2002; Schlüns, Schlüns, Praagh and Moritz, 2003; Wiernasz, Sater, Abell and Cole, 2001), sperm counts were divided by body length.

Ejaculate size

For ‘mating permitted’ trials, orchids were examined with a hand lens for spermatophores. If a spermatophore was visible, it was immediately removed from the flower with forceps and placed on a slide. In situ, the spermatophore was broken on the slide with forceps and treated as per above methods, then later stained and counted.

For some observations it was not possible to estimate the number of spermatozoa: the sperm mass was not spread thoroughly before drying, and flagella stuck together such that individual sperm cells could not be isolated and counted. These observations ($n = 34$) were removed from multivariate analyses, but included in univariate models that did not have sperm counts as a variable. Sperm counts in ejaculate were divided by body length to account for variation in male size.

Statistical analysis

Differences in morphology, sperm use, and behaviour between sites with and without orchids sites were assessed using a Principal Component Analysis (PCA) followed by MANOVA (S. Fig. 4), using F-values from univariate two-way ANOVAs to determine the relative importance of variables (S. Table 1). Only these variables were used in subsequent analyses.

In R, I created three generalised linear models to determine the effect of antennae length and presence of natural populations of orchids on (1) time til first male arrival; (2) mating duration and (3) ejaculate size. The log of each of these dependent variables was taken for the models to meet assumptions of normality. Site was included as a variable in these models to account for any differences arising from inadequate site-matching.

The effect of antennae length and the presence of natural populations of orchids on binary behavioural variables that could not be tested for in the PCA and MANOVA (but coloured in S. Fig. 4 B and C) were assessed using two binomial generalised linear models. For all statistical models, all p values were adjusted for multiple comparisons using the Benjamini and Hochberg correction.

For all models, I calculated Cohen's f^2 as an estimate of effect size (Selya, Rose, Dierker, Hedeker and Mermelstein, 2012). This is equivalent to $\frac{R^2}{(1-R)}$ such that $f^2 \geq 0.02$, $f^2 \geq 0.15$, and $f^2 \geq 0.35$ indicate small, medium, and large effect sizes.

Question 2: Modelling the persistence of haplodiploid or diploid populations in the face of sexually deceptive orchids.

Mating events

Our model tracks the population dynamics of either a haploid or a diploid population of pollinators. Model variables and symbols are given in (S. Table 3). I assume that there are D orchids within a unit area of habitat. Here, 'unit' is defined as the area required to sustain K pollinators (measured at the beginning of the season, i.e. all the recruited offspring from the previous season). In the absence of orchids or sex ratio biases, this area will contain $K/2$ females and $K/2$ male pollinators at the beginning of the season. Note that I use the term 'pollinator' to refer to individuals of either

sex, though only males actively contribute to pollination. These results apply regardless of the value of K since it is a parameter that scales what is considered to be the unit area. There being D orchids per wasp consequently applies across all values of K (our examples use $K = 2$).

I track the population over T_{\max} generations, where every generation occurs over a season of length S days, and each day is split into 100 time steps (thus the total season length is $100S$ time steps). Dividing days into 100 steps is designed to have each time step short enough to represent a discretized approximation (Euler method) of differential equations, where each step can be assumed to yield maximally one encounter for each focal individual (valid across all reasonable values of male search efficiency). T_{\max} is chosen to be large enough ($T_{\max} = 50$) such that the population always stabilizes or goes extinct (in practice, since this model is deterministic, these happen quickly, in 20 generations or less). Generations are non-overlapping.

At the beginning of each generation (first time step of the first day of the season), males emerge. Since I need to track the mating status of the males, I assume a male mating capacity of c matings before becoming sperm depleted. The population is initialized with a density of $M_0(t) = K/2$ males at $t = 1$, with the subscript 0 indicating the male's mating status, i.e. the number of times they have mated so far. All other $M_i(t)$ values, for $i = 1, 2, \dots, c$, are set to 0, as all males are initially in a virgin state. If a male of category c mates again, I assume him to stay in category c ,

since there is no need to track the precise numbers of matings for a male who is already sperm depleted. ‘Matings’ with orchids count towards a male’s mating status, since these deplete his sperm just like matings with females do (I assume males do not ‘learn’ to avoid approaching or ejaculating on orchids).

Females (F) are initialized using a similar procedure, but with two differences: protandry makes them arrive later, and I need to track not only how many times females have mated (0 or 1, due to my assumption of protandry) but also whether they have received sperm (0 or 1). Note that it is possible to have mated but not have received sperm, if the mating happened with a depleted (category c) male. I therefore track the densities $F_{jk}(t)$, where the subscript j refers to the former aspect of female state (0 = virgin, 1 = nonvirgin), and k to the latter (0 = without sperm, 1 = with sperm). This means that $F_{01}(t)$ females cannot exist for any t , and I do not keep track of this variable. All other $F_{jk}(t)$ are set to zero until the emergence time point $t = 100T_P + 1$, where T_P measures the length of protandry in days. For example, if $T_P = 2$, then females emerge at $t = 201$, i.e. 200 time steps (two full days) after the male emergence time $t = 1$. At this t , I set $F_{00}(t) = K/2$ and keep the other $F_{jk}(t)$ as zero.

At each time step ($t = 1$ onwards), all males search with search efficiency s . The number of encounters between a male of mating category i and a female of category 00 is

$$C_i(t) = s M_i(t) F_{00}(t) dt,$$

where dt is set to $1/100$ in accordance of my decision to split one day being into 100 time steps, and the letter C is used as these encounters are between conspecifics.

Note that all $C_i(t)$ values are zero before female emergence, and that males do not mate with other categories of females than 00 (since I assume monandry). Males also mate with orchids (at all t , i.e. both before and after females have emerged), and encounter them at rate

$$H_i(t) = s M_i(t) D dt$$

where the letter H is used to denote heterospecific encounters.

The temporal dynamics is then computed, for males, as

$$M_i(t+1) = M_i(t) - C_i(t) - H_i(t) + C_{i-1}(t) + H_{i-1}(t)$$

This formulation takes into account that males that have mated i times may stop being in this category (the terms $-C_i(t) - H_i(t)$) if they mate either conspecifically (with females) or heterospecifically (with orchids); the number of these males can also increase from the previous matedness class (category $i-1$) if such males mate, again either with females or with orchids (the last two term). The two special categories are $i = 0$, for which I omit the last two terms (there cannot be fewer matings than 0), and $i = c$, for which I omit the two negative terms $-C_i(t) - H_i(t)$, since a sperm depleted male will never stop being sperm depleted (I assume negligible

sperm replenishment, therefore males do not move on from a sperm depleted state to any other).

For females, there are three categories to keep track of: virgin (00) females, mated but without sperm (10), and mated with sperm (11). Matings with non-depleted males bring a female to a mated state, which may or may not also give her sperm:

$$F_{00}(t+1) = F_{00}(t) - \sum_{i=0}^c C_i(t)$$

$$F_{10}(t+1) = F_{10}(t) + C_c(t)$$

$$F_{11}(t+1) = F_{11}(t) + \sum_{i=0}^{c-1} C_i(t)$$

Reproduction

I next track the egg production of females. For practical reasons (I need to compute u , as described below), this is done for each t after the entire sequence $M_i(t)$, $F_{00}(t)$, $F_{10}(t)$ and $F_{11}(t)$ is known for the current season. I assume a delay of T_E days from female emergence to the commencement of egg production, thus egg production is checked from time $t = 100(T_P + T_E) + 1$ onwards. The rules of egg production differ between diploids and haplodiploids. For diploids, only category 11 females (mated, and with sperm) can reproduce. For diploids, half of produced eggs are male (denoted E_M), half are female (denoted E_F). Diploid egg production at time t equals

$$E_M(t) = \begin{cases} \frac{f}{2} F_{11}(t) & t \geq 100(T_P + T_E) + 1 \\ 0 & \text{otherwise} \end{cases}$$

$$E_F(t) = \begin{cases} \frac{f}{2} F_{11}(t) & t \geq 100(T_P + T_E) + 1 \\ 0 & \text{otherwise} \end{cases}$$

Here f is the egg-laying rate.

For haplodiploids, any female can contribute to male production, while only mated females with sperm (category 11) can produce daughters. I assume that the sex ratio, r , that the category 11 females choose has evolved to take into account the overall level of spermlessness among females. Averaged over the entire season, the proportion of such mothers is

$$u = \frac{\sum_{t=100(T_P+T_E)+1}^{100S} F_{00}(t) + F_{10}(t)}{\sum_{t=100(T_P+T_E)+1}^{100S} F_{00}(t) + F_{10}(t) + F_{11}(t)}$$

Category 11 females then adopt a sex ratio that comes from the analysis of Rautiala et al (Rautiala et al., 2017).

$$r = \begin{cases} \frac{(1-2u)}{2(1-u)} & \text{if } u < 0.5 \\ 0 & \text{if } u \geq 0.5 \end{cases}$$

The overall egg production for the haplodiploid case takes the form

$$E_M(t) = \begin{cases} f(F_{00}(t) + F_{10}(t) + rF_{11}(t)) & t \geq 100(T_P + T_E) + 1 \\ 0 & \text{otherwise} \end{cases}$$

$$E_F(t) = \begin{cases} (1-r)fF_{11}(t) & t \geq 100(T_P + T_E) + 1 \\ 0 & \text{otherwise} \end{cases}$$

Note that my computation of r is based on females having evolved to cope with the currently valid level of spermlessness, u . At evolutionary equilibrium, which is the solution the dynamic is converging to, this can be expected to apply accurately.

Before that, when u undergoes transient changes, this is best seen to be an approximation. I always report equilibrium values in the main text and figures.

In both cases (diploid and haplodiploid), the next generation is initiated with M females and F females. While the initial population densities were set to $K/2$ of either sex as explained above, the new generation of recruits is computed as

$$M = \begin{cases} \sum_t E_M(t) & \text{if } \sum_t (E_M(t) + E_F(t)) \leq K \\ \sum_t E_M(t) \frac{K}{\sum_t (E_M(t) + E_F(t))} & \text{if } \sum_t (E_M(t) + E_F(t)) > K \end{cases}$$

$$F = \begin{cases} \sum_t E_F(t) & \text{if } \sum_t (E_M(t) + E_F(t)) \leq K \\ \sum_t E_F(t) \frac{K}{\sum_t (E_M(t) + E_F(t))} & \text{if } \sum_t (E_M(t) + E_F(t)) > K \end{cases}$$

Here, the sum is applied over all t belonging to the current season (1, 2, ..., 100 for season 1, 101, 102, ..., 200 for season 2, etc). The number of recruits is computed considering that the total population (males+females) cannot exceed K per unit area, while too low productivity means that the new generation may start with fewer than K pollinators. The recruits M and F form the M_0 and F_0 values at the appropriate t , as explained above.

Museum and digital record survey

In both Australia and New Zealand, *L. excelsa* is distributed broadly, while *Cryptostylis* orchids occur in patchy locations. I recorded the sex and location of *L. excelsa* specimens from entomological collections from museums and digital records from social networks (e.g. iNaturalist) across Australia and New Zealand (n = 626). For comparison, I identified and recorded the sex and location of a sister ichneumonid species, *Echthromorpha intricatoria*, which lives in the same areas (with and without orchids) as *L. excelsa* and shares a host (typically *Helicoverpa* larvae; n

= 191 ;Berry and Walker, 2004). To my knowledge, *E. intricatoria* has never been recorded as a pollinator of sexually deceptive orchids. I therefore expect that the presence of orchids would not affect their adult sex ratio. The presence of orchids was determined using records from the Atlas of Living Australia and Herbarium records (n = 4,044).

Many of the records' localities were not specific and coordinates were not provided. As such, I used approximate coordinates found using the written locations. Where locations were too broad (e.g. "Sydney" versus "Macquarie"), they were excluded from the analysis. To avoid collection bias, I excluded male wasps that were found on orchids, had orchid wishbone pollen attached to their abdomen, or were collected by orchid researchers. Similarly, I excluded females found in agricultural locations to avoid bias in collectors interested in hosts.

Where possible, I recorded month and year of collection. Because very few years had multiple observations within them, they were lumped into decades. I carried out an initial generalised linear model to detect bias in sampling over time, i.e. whether sexes were more likely to be caught in a decade or month. This revealed that between June and October, there were no records of males for either *L. excelsa* or *E. intricatoria*. As male ichneumonids do not live as long as females, and may only be present during mating season (Quicke, 2014), I excluded observations outside of the mating season from subsequent cluster analyses.

In order to determine whether records came from sites with or without orchids, I used the Geosphere package in R (Hijmans, 2019) to carry out hierarchical cluster analysis based on the latitude and longitude of each observation. Every observation was clustered into a group based on their distance from one another, such that all observations in a group were within 15km radius of each other. A group was defined as “with orchids” if it was within 15km of an orchid observation. Finally, sex ratio was only analysed for groups with 4 or more observations, and for groups where the sex ratio was greater than 0 and less than 1.

Ultimately, my observations were reduced to 130 records of *E. intricatoria* (n (groups with) = 3; n (groups without) = 7) and 326 records of *L. excelsa* (n (groups with) = 10; n (groups without) = 23).

I calculated the sex ratio for each group of specimens and carried out a generalized linear model using a binomial distribution to determine whether there was a difference in sex ratio between species at sites with and without orchids, blocking by country. Sex ratio was weighted by the number of observations in each group.

Multiple comparisons were accounted for using Benjamini and Hochberg corrections.

2.9 Supplementary tables and figures

S. Table 1 A MANOVA test for differences between sites with and without orchids for measured variables was significant. Subsequent multiple ANOVAs revealed the main variables responsible for these differences were time til first male arrival; ejaculate size; antennae length and mating duration (larger F values indicate importance; p value from ANOVA not used as inflates type I error).

Variable	ANOVA F value	MANOVA p value
Vesicle area	0.1	
Testes area	1.0	
Sperm stock	1.8	
Interaction duration	5.6	
Eye area	6.9	
Body length	9.8	
First male arrival	31.5	< 0.0001
Ejaculate size	36.0	
Antennae length	39.8	
Mating duration	59.5	

S. Table 2 : Total number of trials at each site as well as the number of failed trails (where no males came in to mate with an orchid).

Orchid presence	Site number	Total 'failed trials'	Total number of trials	'Fail' rate (%)	Fisher's exact test p value
Absent	1	15	39	38.5	> 0.5
	2	11	31	35.5	
	3	5	27	18.5	
Present	4	16	38	42.1	
	5	8	28	28.6	
	6	20	38	52.6	
Total		75	201	37.3	

S. Table 3 Definitions for model variables

Symbol	Definition
K	The number of pollinators (male and female) that can be sustained in a unit area; = 2 for all simulations
T_{\max}	The total number of generations; = 50 for all simulations.
S	Season length; = 50 for all simulations (unless stated otherwise)
c	Male mating capacity; = 30 for all simulations (unless stated otherwise)
D	Orchid density; the number of orchids in an area for every one female
s	Search efficiency
f	Egg laying rate
T_P	Length of protandry in days
T_E	Number of days since female emergence that egg production begins; = 1 (unless stated otherwise)

S. Table 4 Site matching information for three chosen sites with and without natural populations of orchids. Pollinator, pollinator host and orchid abundance was first assessed using Atlas of Living Australia (ALA) and then with subsequent transects and sampling at each site. Number of flowers on orchids and pollination rates were assessed over the course of the field studies (2016 – 2018)

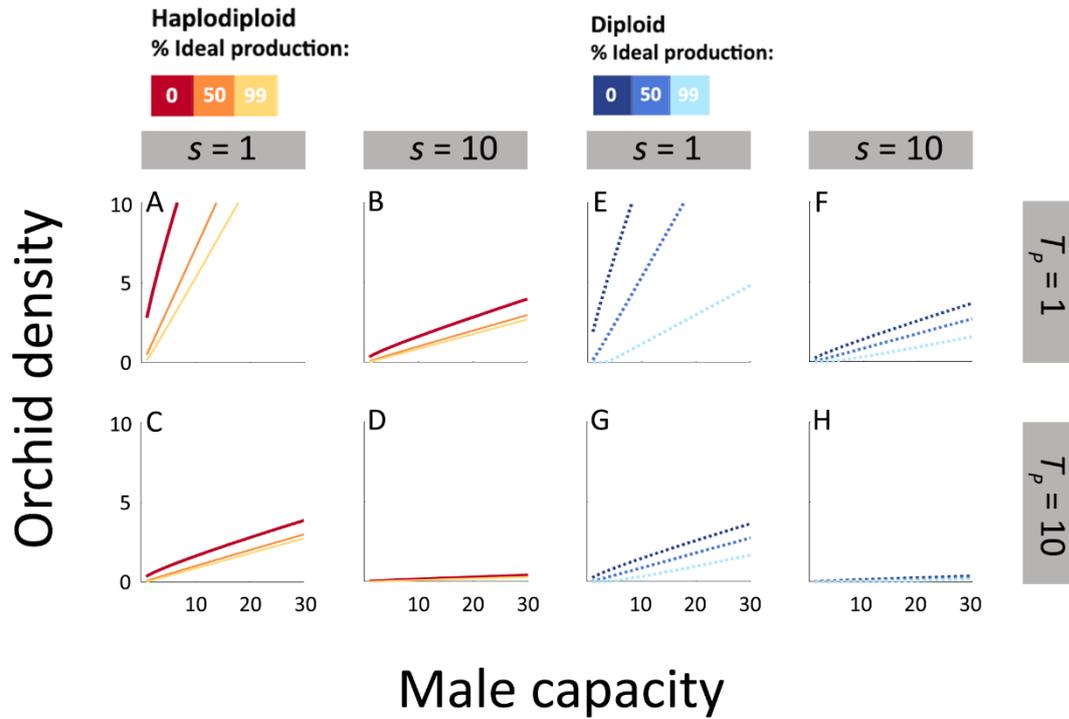
Orchid presence	Site code	Eucalyptus present	Vegetation type	Pollinator abundance (ALA)	Orchid abundance (ALA)	Helicoverpa abundance (ALA)
Site with orchids	MQ	Yes	Field against native bush	3	108	3
	AO	Yes	Field against native bush	2	93	1
	NFG	Yes	Field against native bush	2	86	2
Site without orchids	ASH	Yes	Field against planted bush	2	-	1
	MK	Yes	Field against planted bush	2	-	1
	LP	Yes	Field against planted bush	1	-	2

S. Table 4 cont.

Orchid presence	Site code	Captured pollinators (sticky traps)	Plants (1 leaf per plant; year 1)	Plants (1 leaf per plant; year 2)	Flowers (all plants, all racemes; year 1)	Flowers (all plants, all racemes; year 2)	Flowers pollinated (year 1)	Flowers pollinated (year 2)
Site with orchids	MQ	2	379	321	219	196	113	102
	AO	0	309	288	181	202	136	152
	NFG	1	340	334	228	126	55	86
Site without orchids	ASH	1	-	-	-	-	-	-
	MK	2	-	-	-	-	-	-
	LP	1	-	-	-	-	-	-

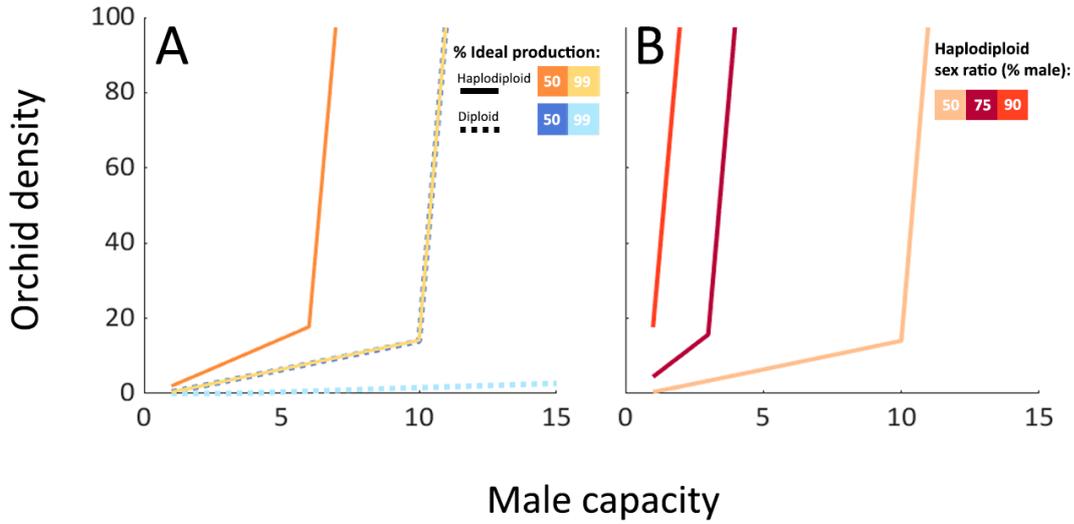
S. Table 4 cont.

Orchid presence	Site code	Pollination rate (year 1)	Pollination rate (year 2)	Soil – Clay classification	Soil – Water classification	Soil – Nitrogen classification	Soil – Phosphorous classification
Site with orchids	MQ	0.52	0.52	0.1	0.5	0.05	0.02
	AO	0.75	0.75	0.1	0.5	0.05	0.02
	NFG	0.24	0.68	0.1	0.5	0.05	0.02
Site without orchids	ASH	-	-	0.1	0.5	0.05	0.02
	MK	-	-	0.1	0.5	0.05	0.02
	LP	-	-	0.1	0.5	0.05	0.02



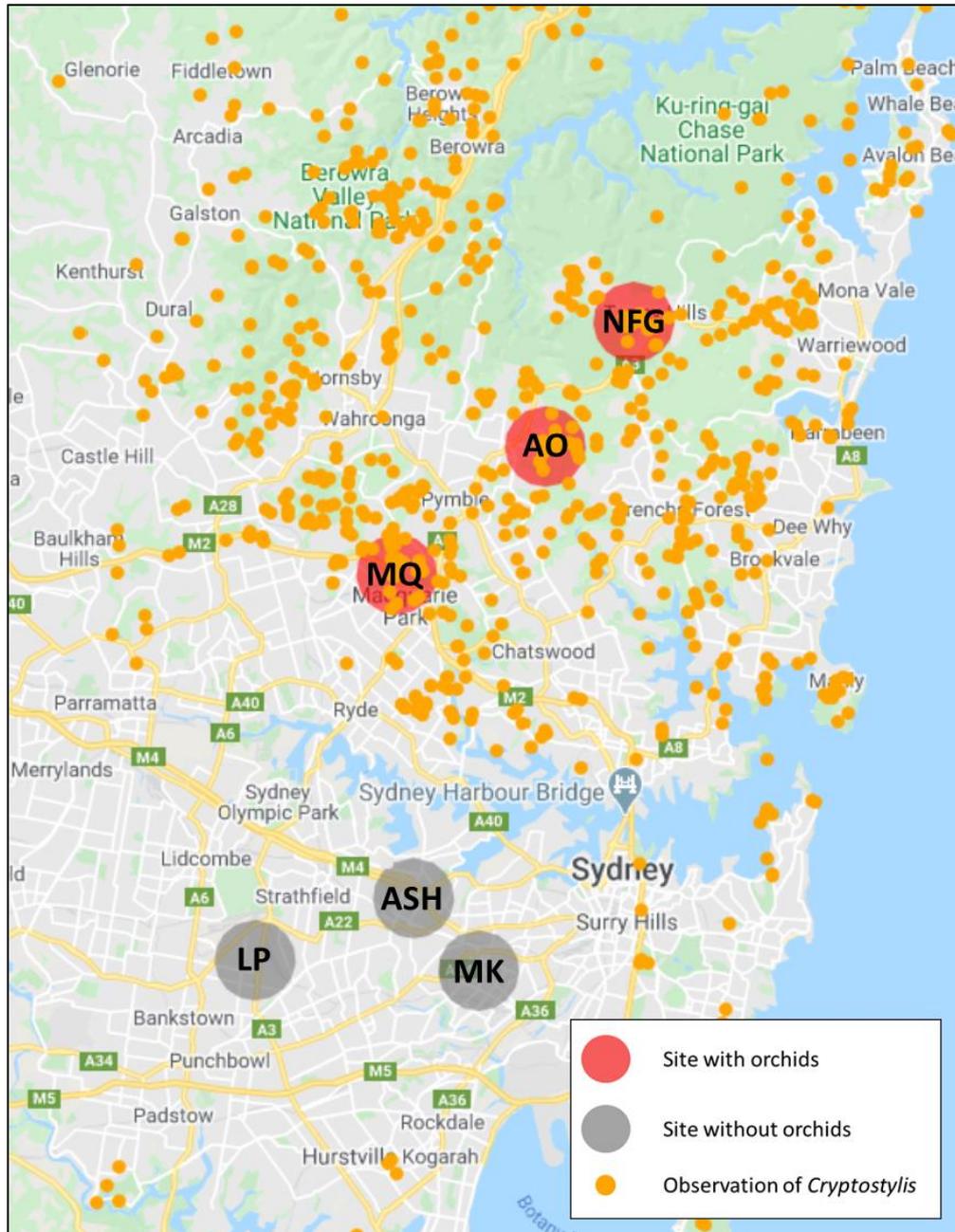
S. Fig. 1 Daughter production of haplodiploid (left; red/orange/yellow) and diploid (right; blues) populations for decreasing levels of T_P and s , while f remains constant at 1. Orchid density (D) reflects the number of orchids in the environment per true females, male capacity (c) is the number of times a male can mate before he becomes sperm depleted. Curves indicate the threshold orchid density that permits 0, 50% or 99% of the production level of the best-case scenario to be maintained, with best-case computed within each panel as daughters produced per female when $c = 30$ and $D = 0$.

A/E: $s = 10$, $T_P = 10$; B/F: $s = 10$, $T_P = 1$; C/G: $s = 1$, $T_P = 10$; D/H: $s = 1$, $T_P = 1$

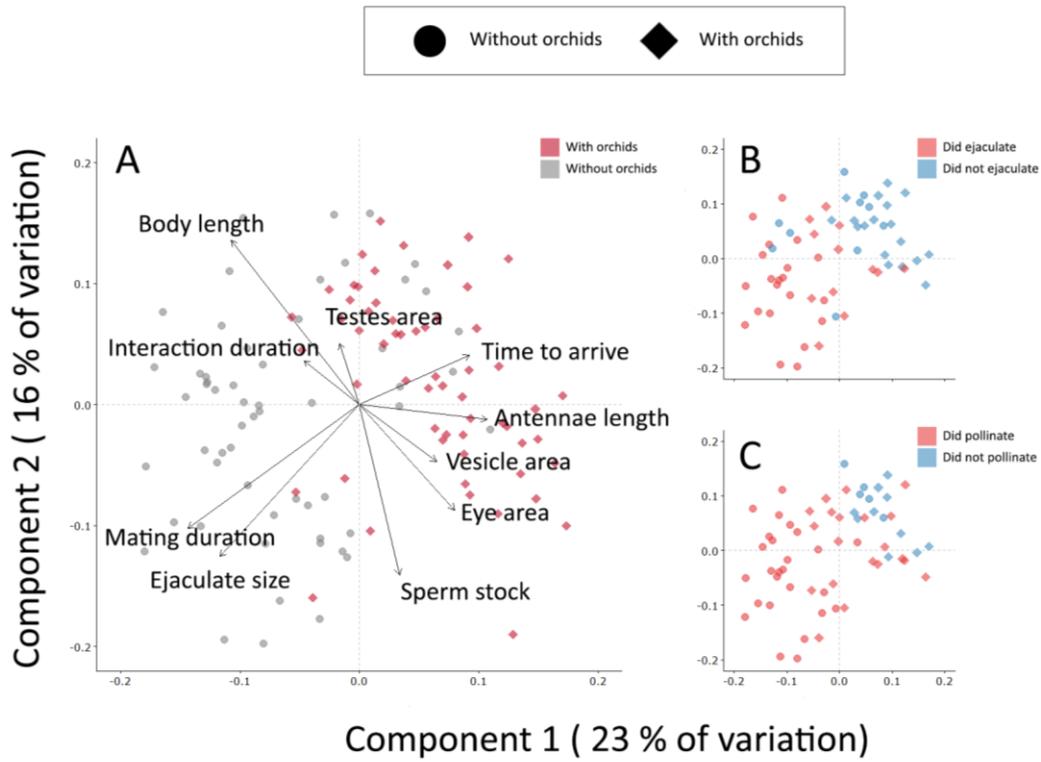


S. Fig. 2 (A) Daughter production of haplodiploid and diploid populations. Curves indicate the threshold orchid density that permits 50% or 99% of the production level of the best-case scenario to be maintained, with best-case computed within each panel as daughters produced per female when $c = 30$ and $D = 0$. Note that no extinction takes place (0% of production level).

(B) Sex ratio predictions. Diploid populations do not adjust sex ratios as there is no production of males caused by matelessness; for these, only the location of the persistence threshold is given, sex ratio is 1:1 everywhere below the persistence threshold, and the population is extinct above it. For haplodiploids, sex ratios are 1:1 underneath the pale orange curve, below 75% underneath the burgundy curve, below 90% underneath the red curve. Note that no extinction (100% male) takes place. $f = 1$; $T_P = 0$; $s = 1$.



S. Fig. 3 Location of sites with (red) and without (grey) natural populations of *Cryptostylis* orchids used in study. Red and grey circles have a radius of 2km, orange circles indicate an observation of a *Cryptostylis* orchid from either Atlas of Living Australia or herbarium records. Note that sites with orchids are contiguous to national parks, whereas sites without orchids are in planted areas – sites without orchids that were contiguous to national parks were not able to be found. Labels on site circles indicate site code as per S. Table 4)



S. Fig. 4 Principal components analysis comparing males from sites with and without orchids (A) coloured by whether males ejaculated on the orchid (B) or pollinated the orchid (C).

Chapter 3: *Cryptostylis* deceit changes pollinator morphology and behaviour.

Formatted for Functional Ecology

3.1 Introduction

Pollinators are able to exert considerable selection on floral traits. Their behaviours, diversity and sensory perception can change floral scent, colour and shape (Schiestl, 2005; Bohman et al., 2016; Ayasse et al., 2011). However, there is currently little data to indicate whether flowers, particularly deceptive flowers, change pollinator fitness sufficiently to drive reciprocal changes in pollinators. This may be because the costs or benefits of the interaction are small or rare enough so as not to exert influence on pollinator evolution (Jersáková et al., 2006). However, this may also be due to a lack of focus on pollinator responses in the literature (Gaskett, 2011).

Deceptive orchids are an excellent example of flowers that may exert negative impacts on individual pollinators. For instance, sexual deception is a common strategy in orchids - approximately 400 species of orchids are sexually deceptive (Gaskett, 2011; Schiestl et al., 2000), as are one species of daisy (Asteraceae;

Gorteria diffusa; Ellis and Johnson, 2010), and an iris (Iridaceae; *Iris paradoxa*; Vereecken et al., 2012). Sexually deceptive organisms take advantage of other species' mating behaviours by mimicking species-specific mating signals, exploiting their innate biases for certain chemical, visual or tactile cues (Peakall and Handel, 2013; Schiestl et al., 2003; Spaethe, Moser, and Paulus, 2007). Costs to individuals tricked into pollinating sexually deceptive plants have thus far explored whether deception results in reduced mating opportunities. Males will fail to find females among false signals (Jager and Ellis, 2014; Wong, Salzmann, and Schiestl, 2004; Wong and Schiestl, 2002) and females compete with orchids for males, as their mating season coincides with orchid flowering (Chapter 6; Gaskett, Winnick, and Herberstein, 2008).

In 2008, Gaskett et al., observed a novel cost: pollinator ejaculation in response to a floral stimulus (Gaskett et al., 2008). Sexually deceptive tongue orchids (*Cryptostylis* spp.) frequently incited their sole pollinator, the male orchid dupe wasp (*Lissopimpla excelsa*, Family: Ichneumonidae, subfamily: Pimplinae) to ejaculate onto their flowers during pollination. Sperm loss can be an acute cost to solitary parasitoid wasps, as it can result in either temporary or permanent sperm limitation for several species (Boivin, Jacob and Damiens, 2005; Damiens and Boivin, 2006; Jager and Ellis, 2014; Olsson, Madsen and Shine, 1997). In *Lissopimpla excelsa*, males may become at least temporarily sperm depleted, and they will waste approximately 10% of their sperm

with each orchid encounter (Brunton Martin, Gaskett and O’Hanlon, 2020). Despite this, sexually deceptive orchids persist and thrive: sexual deception has evolved several times independently (Herberstein, Baldwin and Gaskett, 2014) and extreme deceivers, such as *Cryptostylis* orchids, have relatively high fitness (Gaskett, 2011). If orchids impose such costs and still maintain extraordinarily high pollination success (up to 90% vs. 30% global mean for other sexually deceptive orchids; Gaskett, 2011), might we expect counter-adaptations to arise in response to deception?

If *L. excelsa* is harmed by the extreme sexual deceit of *Cryptostylis* orchids through sperm loss and depletion (Brunton Martin et al., 2020), I might expect selection to favour males better able to avoid or tolerate sexual deception. Avoidance could involve selection for traits that allow males to distinguish between orchid flowers and true females. For instance, longer antennae in moths, cockroaches, crickets, beetles and water-lice allow animals to better detect and orient to females (Bertin and Cézilly, 2003; Hanks, Millar and Paine, 1996; Johnson, Symonds and Elgar, 2017; Khadka, Shek, Hoffman, Vulin and Foellmer, 2012; Lockety and Willis, 2015).

Similarly, in several species, males have larger eyes compared to females, which may be important in mating encounters (Rutowski, 2000; Streinzer, Brockmann, Nagaraja and Spaethe, 2013). To avoid orchids, I might therefore expect males to have longer antennae or larger eyes (but perhaps not both as a trade-off between neighbouring morphological structures might exist (Emlen, 2001). If males reduce the time spent

with an orchid or the sperm they allocate to an orchid, this may allow males to tolerate sperm loss to orchids (Barbosa, 2011; Kumano, Kuriwada, Shiromoto, Haraguchi and Kohama, 2010). I have previously shown that male *L. excelsa* vary their ejaculate size in response to sexual deception (Brunton Martin et al., 2020), but this is also apparent in other species: in *Drosophila*, males respond differently to sperm competition to maximise fitness. When sperm competition is high, males will decrease copulation duration. On the other hand, when the risk of sperm competition is high, males will increase copulation duration (Bretman, Fricke and Chapman, 2009). Males may also allocate sperm differently to females depending on perceived female quality, mainly when males are prone to sperm depletion (Reinhold, Kurtz and Engqvist, 2002). In invertebrates, males will vary absolute numbers of sperm and number of viable sperm depending on female quality or virgin status (Engqvist and Sauer, 2001; Thomas and Simmons, 2007; Wedell and Cook, 1999).

Alternatively, any differences in male morphology or behaviour may not be a consequence of selection from orchids, but instead a result of the changes orchids exert on the deceived population. By comparing diploid and haplodiploid pollinators, my previous model found that haplodiploids, such as *L. excelsa*, are less prone to extinction in the face of intense sexual deception than diploids (Chapter 5). They persist, in part, because they are better able to cope with sperm limitation through a shift in sex ratio. When orchids elicit sperm wastage, they produce a shortage of

sperm that drives a high proportion of females to be sperm limited and, as they are haplodiploid, constrained to produce only sons (Rautiala, Helanterä and Puurtinen, 2017). If deception creates an surge in male insects, it could lead to a change males' general behaviour and morphology in the population. Ichneumonids typically exhibit scramble competition, in which males emerge early and patrol for females who, upon emergence, release sex pheromones that quickly attract many males who compete to be her mate (Quicke, 2014). An increase in males could intensify the effects of scramble competition, and I might expect to see the associated traits: larger sensory structures; smaller body size; faster response times and shorter mating durations (Herberstein, Painting and Holwell, 2017).

Here, I test whether responses to sexual deception arise, either in the form of counter-adaptations or enhanced scramble competition. Holding impacts of sexual selection constant, I broadly compare behaviour and morphology of their sole pollinator, *L. excelsa*, between sites with and without selection pressure from orchid deceit. Morphological differences are also compared to behaviour, to determine what the purpose of any difference may be. I expect that under the counter-adaptation hypothesis, males with larger sensory structures will have improved discriminatory ability. In contrast, under the scramble competition hypothesis, they will be more likely to mate. Similarly, I would expect larger sperm stocks under the counter-adaptation hypothesis, associated with reduced sperm use.

3.2 Methods

Field trials and site selection

I tested for differences in morphology and behaviour of male *L. excelsa* that are either frequently deceived, or never deceived, by comparing three sites with and three sites without natural populations of orchids in Sydney, Australia (chapter 2, S. Fig. 3). I used museum and herbarium (Australian Museum, CSIRO, ANIC, Atlas of Living Australia) records to classify these sites as locations with wasps present and orchids either absent or present. I confirmed these classifications before the first field season in September 2016, where I used several 10m transects to explore for orchids thoroughly. Sites were at least 2km apart, exceeding the maximum dispersal range of *L. excelsa* (700m ; Weinstein, Davis, Menz, Dixon and Phillips, 2016). Sites were matched as per the methods laid out in chapter 2 (see S. Table 4)

Between December and February 2016-2018, I recorded pollinator behaviour in 15-minute field trials (as per Bower and Brown, 1997): an unpollinated flower of either *C. subulata* or *C. erecta* was exposed in the field, in an insect rearing cage, until a single pollinator visited. As I used this data in another study that estimated sperm availability in populations (Brunton Martin et al., 2020), males were randomly assigned to a ‘mating permitted’ or ‘mating prevented’ treatment before the commencement of each trial. Pollinators in the ‘mating prevented’ treatment were

caught outside of the rearing cage as they approached the orchid. Pollinators in the ‘mating prevented’ treatment could enter the cage and choose to mate, with their behaviour recorded.

If I did not attract any pollinators after two trials, I removed the orchid and began a new trial 5 metres away. I used orchid flowers within 48 hours of collection and, where possible, re-used flowers used in the ‘mating prevented’ in later trials. After I recorded behaviour, wasps were caught and killed by freezing within 4 hours.

Behaviour

I recorded pollinator behaviour, during ‘mating permitted trials, using a voice recorder and video recorder. Behaviours recorded related to either (1) ease of deception: latency til first-male arrival at an orchid bait, and whether the wasp landed on the orchid or flew past (2) mating effort: copulation duration (associated with sperm transfer; Barbosa, 2011), total interaction time (time spent on orchid copulating and carrying out post-copulatory behaviour), whether males deposit sperm, or whether they demonstrated pre- or post-copulatory behaviours (wing fanning, antennal grooming, abdominal grooming).

Morphology

All males from ‘mating prevented’ and ‘mating permitted treatments were caught and killed by freezing. Morphology related to either (1) detection: eye area, antennal

length; (2) sperm availability: testes area, seminal vesical area; or (3) animal size: abdomen length, body length and abdomen area was photographed with a microscope and measured using ImageJ (Fig. 1). Because morphology varies with animal size, all measurements were divided by body length.

Sperm stock and ejaculate size

I assessed sperm stock following methods by Bressac et al.(2008): following capture, I quickly dissected males and removed their seminal vesicles. Placing them on a slide with saline (128.3nM NaCl*137mM in phosphate buffer pH 7.2) and 0.3% triton solution to avoid flattening. They were then broken open with an entomological pin and gently stirred to disperse the sperm, fixed with ethanol, then left to dry at ambient temperature. I stained these slides for 15 minutes with DAPI (300nM in PBS) and assessed sperm presence and number of spermatozoa using a fluorescence microscope. Spermatozoa counts were estimated by counting precisely ten spermatozoa, estimating the area of those ten sperm, and measuring the area of the sperm mass to obtain the total number of cells in the mass. As sperm number varies with animal size (Bangham, Chapman and Partridge, 2002; Schlüns, Schlüns, Praagh and Moritz, 2003; Wiernasz, Sater, Abell and Cole, 2001), sperm counts were divided by body length. I examined orchids from ‘mating permitted’ trials with a hand lens for spermatophores. If a spermatophore was visible, it was immediately removed from the flower with forceps and placed on a slide. In situ, the spermatophore was broken

on the slide with forceps and treated as per the above methods, then later stained and counted (Fig. 2).

In total, 60 males from sites with orchids were captured and measured and 59 males from sites without orchids. For some observations it was not possible to estimate the number of spermatozoa: the sperm mass was not spread thoroughly before drying, and flagella stuck together such that individual sperm cells could not be isolated and counted. I removed these observations ($n = 34$) from multivariate analyses, but they are included in models and presented results where possible. Sperm counts in ejaculate were divided by body length.

Statistical analysis

I first assessed differences in morphology, sperm use, and behaviour between sites with and without orchids sites using a Principal Component Analysis (PCA) followed by MANOVA (S. Fig. 1) using F-values from univariate ANOVAs to determine the relative importance of variables (S. Table 1). I restricted subsequent analysis to these variables. As males in the ‘mating prevented’ category would not have mating behaviour or ejaculate size recorded, they had missing data. In order to preserve morphological data for the multivariate analysis, as I randomly assigned the ‘mating prevented/permitted’ category, I replaced missing observations with the median

value for their collection site. Median iterated observations used only for the PCA and MANOVA.

In R, I created three generalised linear models to determine the effect of antennae length and presence of natural populations of orchids on (1) time taken til first male arrival; (2) mating duration (for those that chose to mate) and (3) ejaculate size (for those that deposited sperm). The natural log of each of these dependent variables was taken for the models to meet assumptions of normality.

As the effect of binary variables cannot be tested for in PCA and MANOVA I assessed whether the likelihood of ejaculating or pollinating on an orchid, as well as exhibiting pre or post-copulatory behaviour differed between sites with or without orchids using two logistic regressions. As scramble selection predicts the combination of small animals with large sensory structures and *L. excelsa* have a broad range in male size (in this data, between 6mm and 20 mm, see Fig 3. C), I categorised males in to ‘scramble’ (longer than median raw antennae length and shorter than median body length) and ‘non-scramble’ (median or smaller raw antennae length and median or smaller body length). I tested for a difference in the proportion of ‘scramble’ males between sites with and without natural populations of orchids using a logistic regression.

For all statistical models, I adjusted all p values for multiple comparisons using the Benjamini and Hochberg corrections. In each model, I also tested the effect of year of capture, orchid species used for capture and body length. They did not have a significant effect in any of the models and so were removed to improve the models' fit (assessed via lower AIC).

3.3 Results

Morphological differences

Exploratory principal component analysis and MANOVA revealed a significant difference between males from sites with orchids versus males from sites without (p value < 0.01 , S. Table 1). Male morphology only differed in antennae length – males from sites with orchids had longer antennae relative to their body than males from sites without orchids (Fig 3. A). Otherwise, males had similar testes area, seminal vesicle area, eye area and body length. Intriguingly, at sites with orchids, there were significantly more ‘scramble’ males than at sites without orchids (Fig 3. B).

Behavioural differences

Males from sites with orchid differed in almost all behaviour measures. At sites with orchids, there were longer first-male arrival times, males that did arrive spent less time mating with orchids and, when they ejaculated, spent less sperm than males at sites without orchids (Fig. 4 A-C). However, they were just as likely to ejaculate on

the orchid and pollinate the orchid (Fig. 5). Males did not differ in whether they showed pre-or post-copulatory behaviours.

Correlation with sensory structures

As antennae length was the only morphological variable important in separating sites with and without orchids (S. Table 1, S. Fig. 1) I assessed how this correlated with their behaviour. At sites with orchids, shorter first-male arrival times correlated with longer antennae (Fig. 4 D ; p value < 0.001) and males had shorter mating durations (Fig. 4 E; p value < 0.001). However, ejaculate size did not correlate with antennae length at either sites with or without orchids (Fig. 4 F ; p value > 0.05).

At sites with orchids, longer antennae correlates with increased probability of pollination. In contrast, at sites without orchids, longer antennae correlated with decreased probability of pollination (Fig. 5, p value = 0.03). Antennae length did not correlate with the likelihood of males ejaculating on orchids (p value > 0.05).

3.4 Discussion

I have found evidence that suggests changes in morphology and behaviour could arise in response to sexual deceit by *Cryptostylis* orchids. Males at sites with orchids had longer antennae and there were more small males with long antennae. Furthermore, at sites with orchids, first-male arrival times were shorter, males spent less time mating with orchids and ejaculated less sperm on the orchids. These morphological

and behavioural differences observed may arise as either a direct consequence of deception (counter-adaptation) or indirect consequences (arising from scramble competition).

Sexually deceptive orchids attract their pollinators by mimicking female insect sex pheromones and appearance (Gaskett and Herberstein, 2009). I found that pollinators from sites with orchids had longer antennae relative to their body size (difference 0.1mm/mm body length), indicating increased investment in sensory structures compared to pollinators from sites without orchids. In insects, larger antennae is associated with more olfactory receptors, with more receptors allowing improved sensitivity to chemical signals (Chapman, 1982). Experiments show that male ichneumonids use their antennae, rather than their eyes, for mate-searching (Grosch, 1947). Additionally, *L. excelsa* will readily copulate with a simple pin that does not resemble a female insect, as long as it is scented with *Cryptostylis* flower extract (Bohman, Weinstein, Phillips, Peakall and Flematti, 2019).

The pollinators at orchid sites also took longer to respond to the presentation of an orchid, were less likely to mate with it, and when they did mate, they had shorter mating durations. These behavioural changes may be adaptive responses to orchid deception, but they do not correlate to behaviour in a way that I would expect if longer antennae improved discrimination. Instead, antennae length correlated with shorter times to arrive at orchids, shorter mating durations, and an increased

likelihood to pollinate the orchid. Intriguingly, there were no significant correlations with antennae length at sites without orchids.

Instead, I expect these behaviours under scramble competition: a trait observed in most solitary, parasitoid wasps (Quicke, 2014). If sexual deceit eliciting sperm wastage increases the number of males in a population, as my model predicted (Chapter 5), perhaps scramble competition is more intense within populations that live with orchids. Scramble competition typically selects for males with longer antennae for increased detection, and small body size for increased mobility (Herberstein et al., 2017), and here I report a significantly larger proportion of small males with long antennae at sites with orchids than without.

Furthermore, males with longer antennae had shorter first-male arrival times, possibly due to more efficient searching (but not necessarily discriminating). I expect that when females rarely mate, and sperm competition is therefore low (solitary ichneumonid females generally mate only once in their life ;Quicke, 2014), and the number of males with which to compete to find females is high (scramble competition), males should invest in obtaining a high number of mates at the cost of smaller ejaculates (Parker and Pizzari, 2010). These observations of shorter mating durations reflect this – not only at sites with orchids but overall for males that have long antennae.

There was no correlation between antennae length and sperm deposits or likelihood to ejaculate. Indeed, males were just as likely to pollinate and ejaculate on an orchid regardless of whether they had had prior experience with an orchid. Moreover, populations living with and without orchids had the same sperm stock in testes. However, when males from sites with orchids did ejaculate, they deposited less sperm. Smaller deposits may, therefore, be a consequence of pollinators having evolved to have increased antennae length, or the result of pollinators' learned avoidance after exposure to deceptive orchids (Ferdy, Gouyon, Moret and Godelle, 1998; Gaskett, 2011; Weinstein et al., 2016). Males may vary ejaculate allocation, or not mate in response to current cues if they become aware that their mate is an orchid, or simply perceive the orchid as a low-quality female. The males may also be sperm depleted: in this study, I observed three wasps with less than 1000 sperm in their seminal vesicles (who did not leave ejaculate) – indicating they were sperm depleted. When males become sperm depleted, they will continue to mate: transferring very little sperm, or only seminal fluid without sperm (Boivin et al., 2005).

However, because the males analysed are only those that responded to the presentation of an orchid, and differences in habitat between sites with and without orchids (particularly vegetation structure: sites with orchids are natural bush, while sites without orchids are planted), I can only suggest that orchids provoke change in

their pollinators. Future work should sample the pollinator population (males and females) without use of bait to determine whether there are any morphological differences and if sites are matched in pollinator availability. This may also reveal whether costs to the individuals are large enough that females have different morphology – for example, larger sperm storage organs. Malaise traps might prove most useful for collecting this species but are large and difficult to set up for long periods in public areas. Ideally, small, maneuverable traps that rely on the chemical ecology of the species (other than sex pheromones) will be developed in time. Such traps would have the added utility for assessing populations of *L. excelsa*, and other parasitoid species, as biological control agents in crops. Nevertheless, that these significant correlations between antennae and behaviour exist across all sites with orchids does suggest that orchids may exert some pressure on their pollinator populations.

Despite much early work, it is only in the last decade that studies of deception have moved from descriptive to experimental. Studying deception allows us to gain a deeper understanding of not only sensory systems, but also how evolution may operate. I demonstrate that *Cryptostylis* orchids seem to impose costs not previously quantified and in response to these costs, males behave differently and have different morphology. The relationship between the two, however, does not lend itself to the idea that *L. excelsa* is evolving counter-adaptations in response to the costs. Instead,

by manipulating the sex ratio of these species, orchids create males that search more for mates, while being just as eager to mate as a naïve pollinator.

3.5 Tables and figures

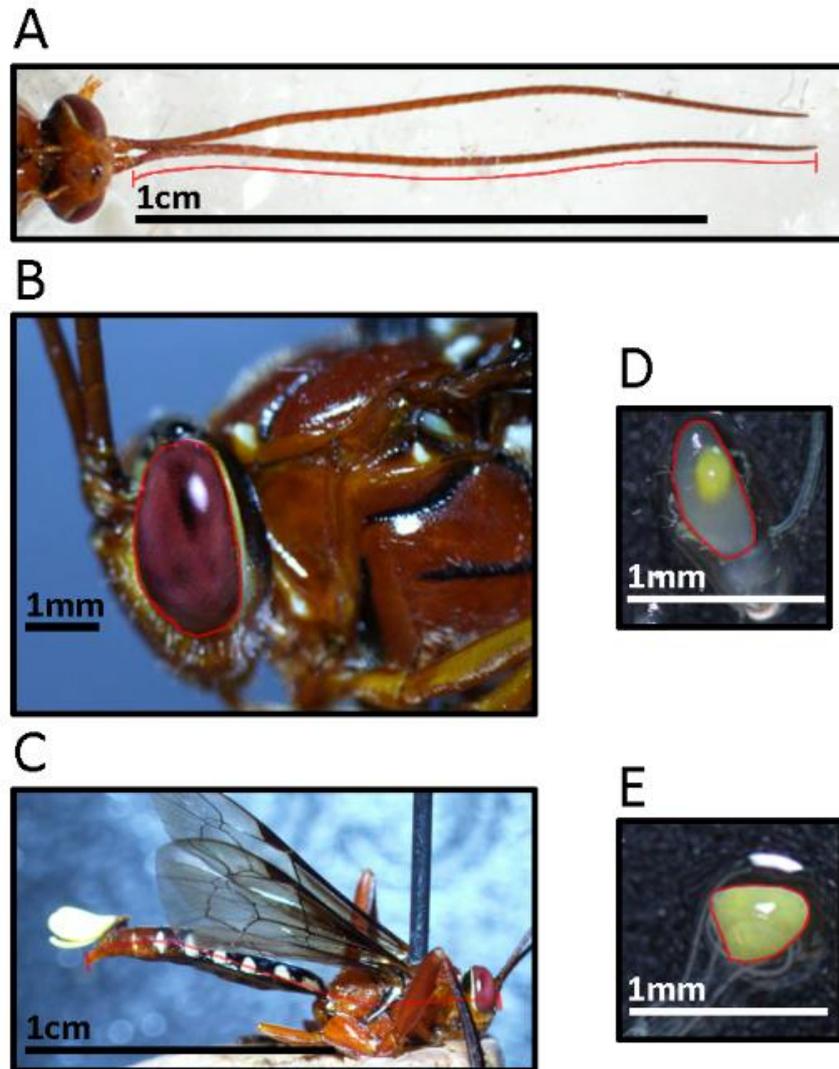


Fig. 1 Morphological measurements taken of captured males from sites with and without natural populations of orchids. Using the magnetic lasso tool in ImageJ (example indicated with red), areas and lengths of various traits were measured and converted from pixels into mm. (A) Antennae length; (B) Eye area; (C) Body length; (D) Seminal vesicle area and (E) Testes area.

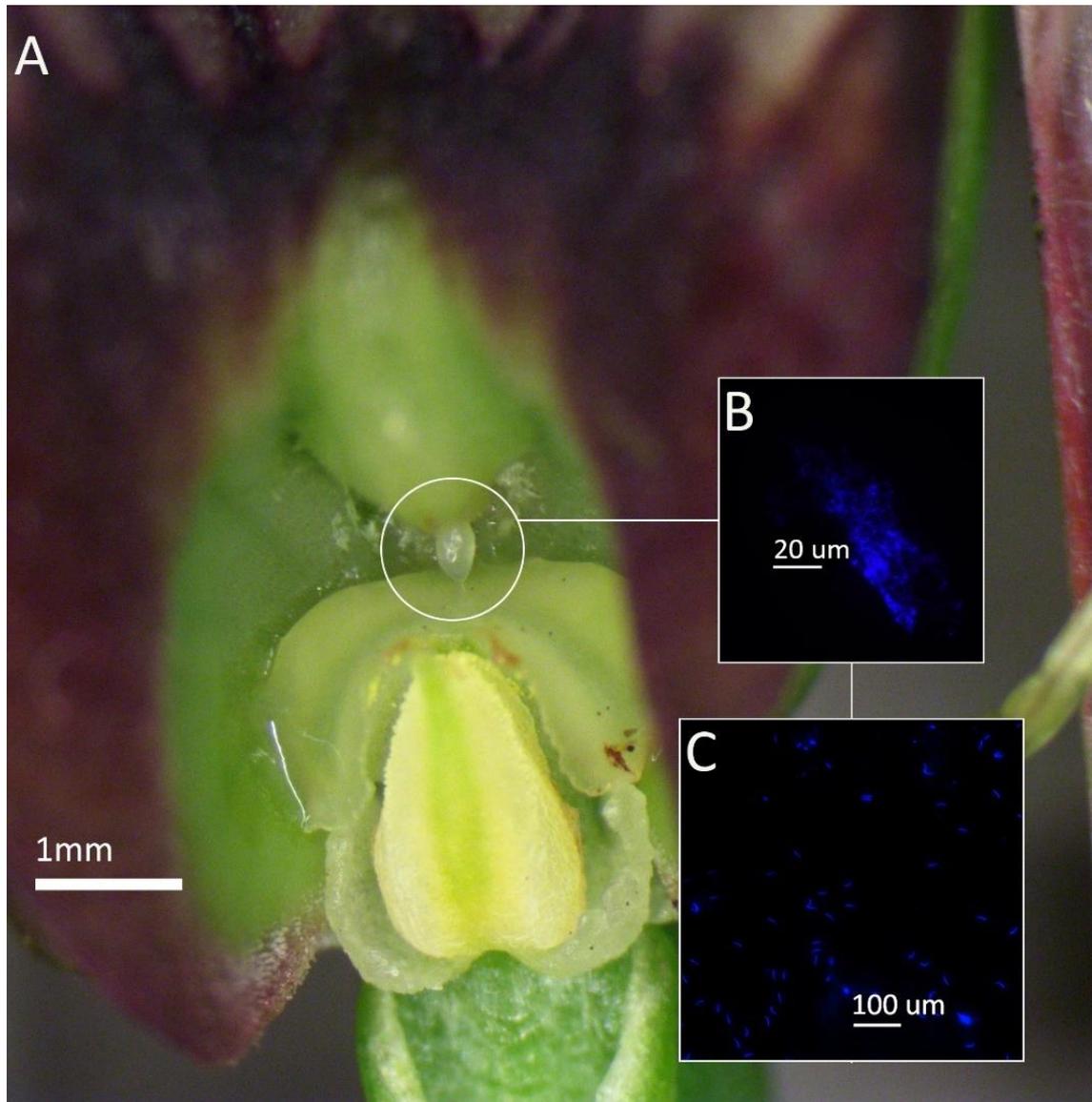


Fig. 2 (A) Spermatophore on *C. erecta* flower, with full sperm mass (B) and individual sperm (C).

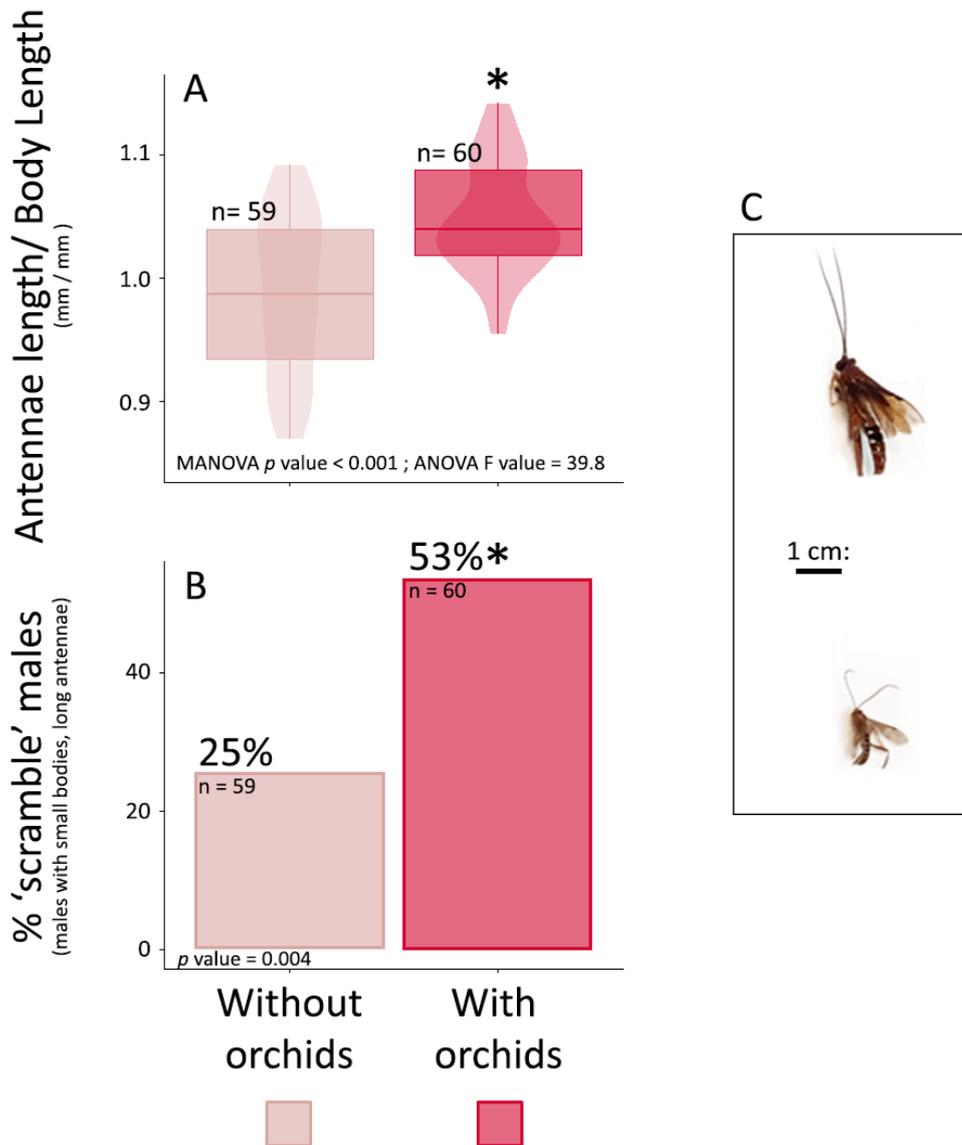


Fig. 3 Males from sites with orchids had longer antennae relative to their bodies than males from sites without orchids (A). In addition, at sites with orchids there are significantly more males that fit a 'scramble' (smaller than median body length, larger than median antennae length) description (smaller than median body length, larger than median antennae length (B)). There is a large range of size in *L. excelsa* males (C).

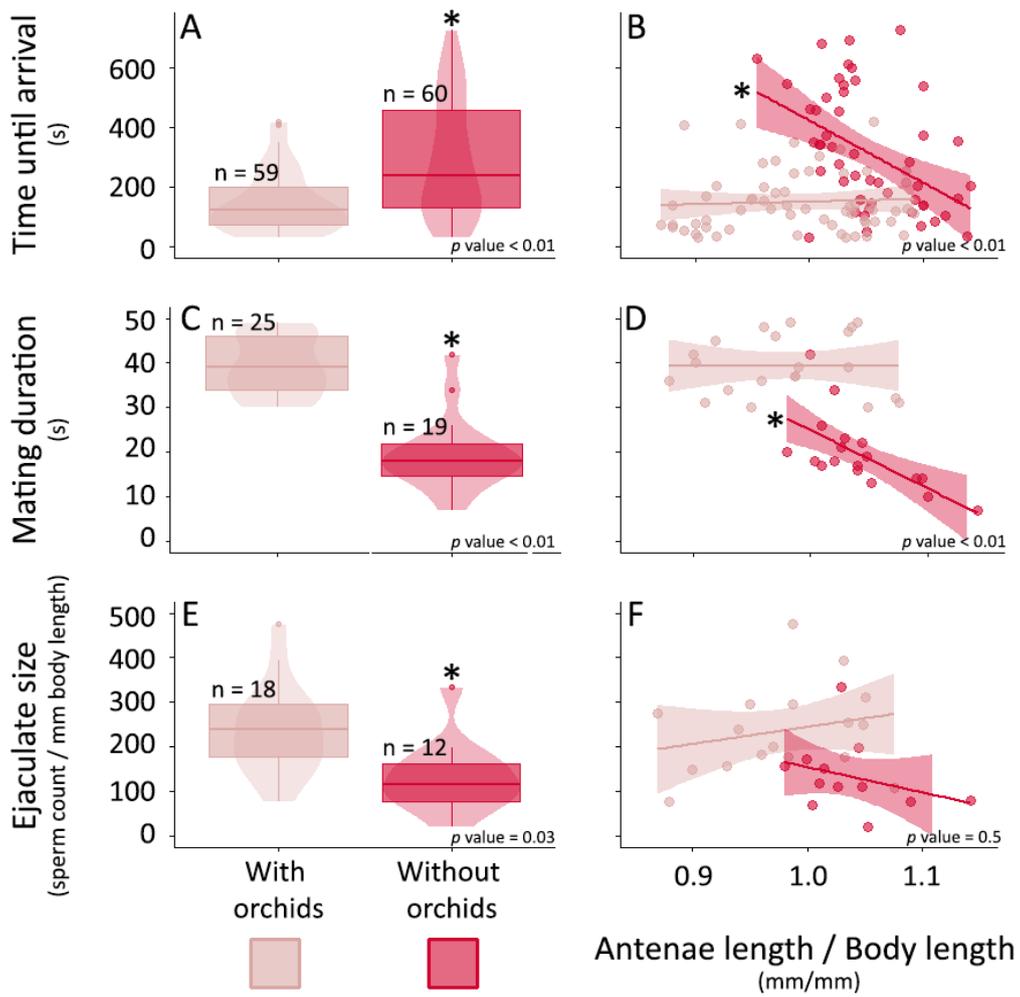


Fig. 4 At sites with orchids, first-male arrival time was longer (A); males shorter mating durations with orchids (B) and, when they ejaculated, deposited less sperm (C) than males from sites without orchids.

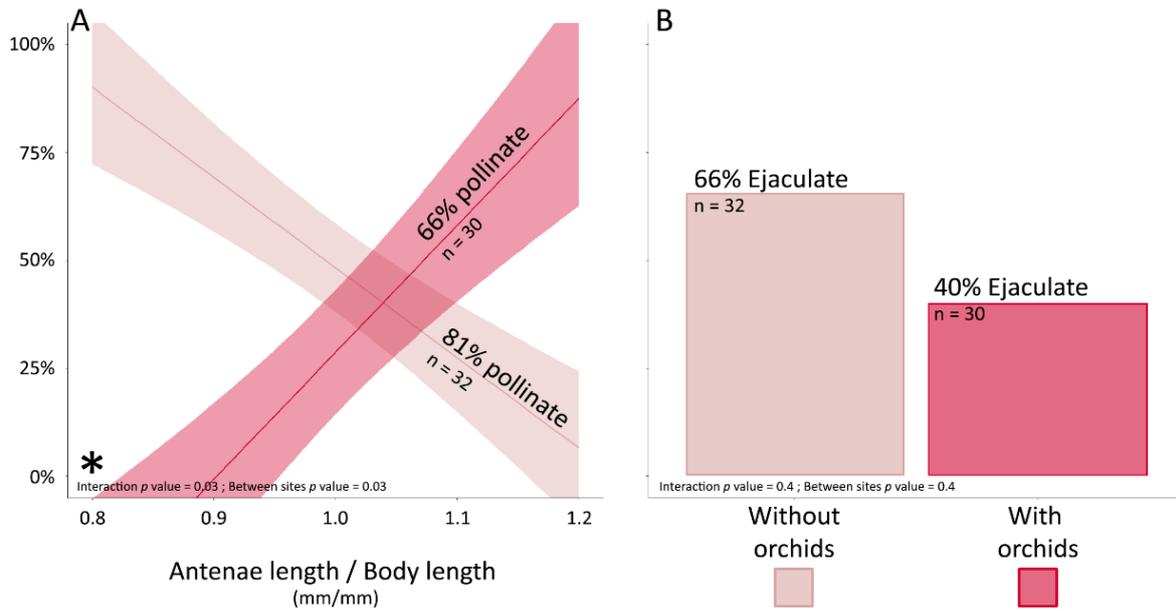
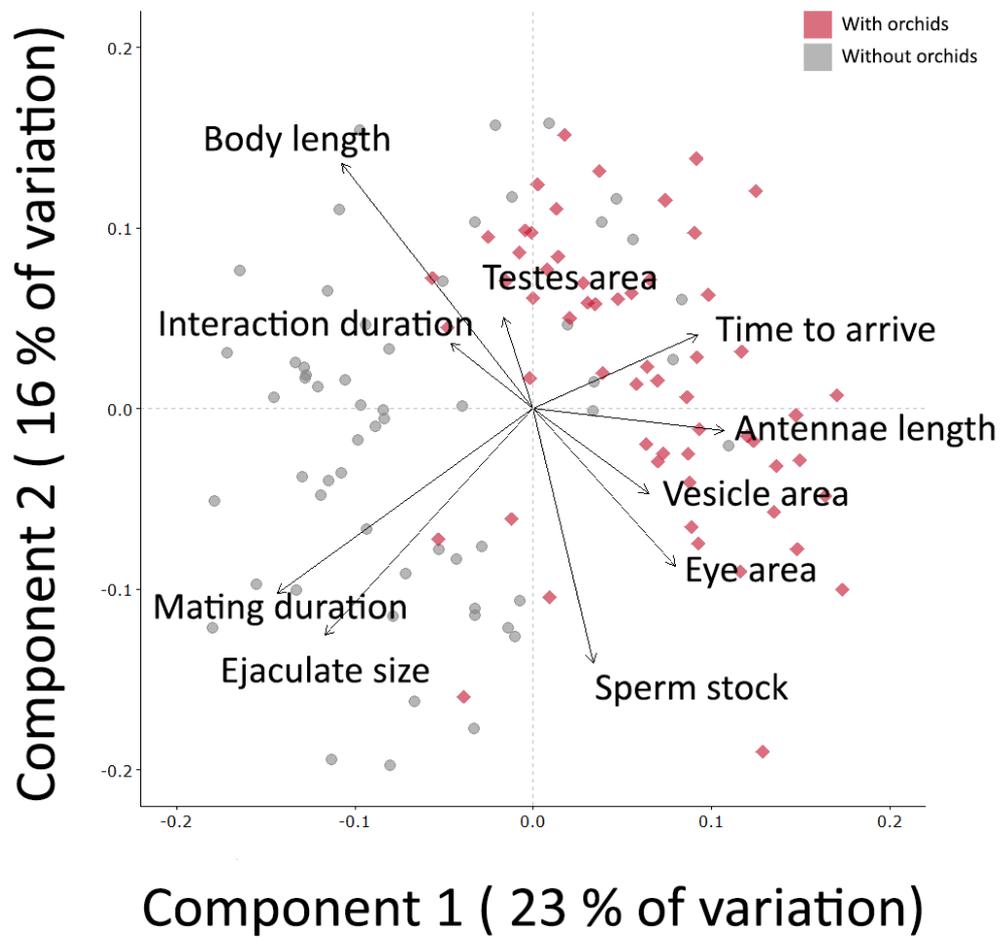


Fig. 5 Males from sites with orchids were just as likely to pollinate an orchid as males from sites without orchids (A). However, at sites with orchids, longer antennae meant that males were more likely to pollinate orchids whereas at sites without orchids, longer antennae meant that males were less likely to pollinate orchids.

3.6 Supplementary tables and figures

S. Table 1: A MANOVA test for differences between sites with and without orchids for measured variables was significant. Subsequent multiple ANOVAs revealed the main variables responsible for these differences were time til first male arrival; ejaculate size; antennae length and mating duration (larger F values indicate importance; p value from ANOVA not used as inflates type I error).

Variable	ANOVA F value	MANOVA <i>p</i> value
Vesicle area	0.1	
Testes area	1.0	
Sperm stock	1.8	
Interaction duration	5.6	
Eye area	6.9	
Body length	9.8	< 0.0001
Time til first arrival	31.5	
Ejaculate size	36.0	
Antennae length	39.8	
Mating duration	59.5	



S. Fig. 1 Principal components analysis comparing males from sites with and without orchids (A) coloured by whether males ejaculated on the orchid (B) or pollinated the orchid (C).

Chapter 4: Orchid sexual deceit affects pollinator sperm transfer.

Published in Functional Ecology

4.1 Introduction

Studies on reproductive fitness often focus on the biology of females – their behaviour, survival and fecundity (Caswell, 2001; Crawley et al., 2017; Helms and Kaspari, 2015; Koch, Phillips, Camus and Dowling, 2018; Özalp and Emre, 2001). Population persistence, however, is impacted by both male and female behaviour (Dyson and Hurst, 2004; Rankin and Kokko, 2007; Wedell, 2010). One critical factor affecting male and female fitness and behaviour is sperm availability. For many species, sperm can become limited, restricting males' ability to mitigate the costs of wasted sperm. Sperm limitation is a problem common to populations with asexual females that still require sperm to reproduce, high levels of sperm competition, or even those that are victims of sexual deceit (Gaskett, Winnick and Herberstein, 2008; Kokko, Heubel and Rankin, 2008; Lehtonen and Schwanz, 2018; Parker and Pizzari, 2010).

Sexual deception occurs when species take advantage of other animals' mating behaviours by mimicking species-specific mating signals, usually exploiting males' innate biases for chemical, visual or tactile cues (Peakall and Handel, 2013; Schiestl et al., 2003; Spaethe, Moser, and Paulus, 2007). Indeed, sexual deceit has been used by humans for sustainable insect control: synthetic pheromone release interferes with communication between sexes and disrupts mating, and lures in insects for mass eradication (Kydonieus, 2019; Witzgall, Kirsch and Cork, 2010). Such lures decrease female mating success, and may increase allee effects that drive populations to extinction (Liebhold and Tobin, 2008). Sexual deceit is a common strategy in orchids (ca. 400 spp. are sexually deceptive; Gaskett, 2011) and can have direct impacts on their pollinators' mating behaviour. For example, deceived males can fail to find females among orchids' false signals (Wong, Salzmann and Schiestl, 2004; Jager and Ellis, 2014; Wong and Schiestl, 2002), and may even waste sperm on the orchid during pollination (Gaskett et al., 2008). It is unknown whether these impose any negative consequences or selection on pollinators because the costs are not yet quantified.

A useful model system for analysing both sperm use and the impact of sexual deception on pollinator reproduction is the Australasian tongue

orchid, *Cryptostylis* spp. (Gaskett and Herberstein, 2009). These orchids mimic the appearance and pheromones of the females of its pollinator, the solitary parasitoid wasp, *Lissopimpla excelsa* (Ichneumonidae; Pimplinae) (Bohman, Weinstein, Phillips, Peakall and Flematti, 2019; Gaskett, 2012; Gaskett and Herberstein, 2006). *Cryptostylis* orchids achieve pollination by fooling male *L. excelsa* wasps into mating with their flowers (Figure 1). During most pollination visits, the male wasps will even ejaculate and waste sperm on the orchid (about 70% of interactions; Gaskett, Winnick and Herberstein, 2008). This sperm wastage may impose costs on males that mate with flowers, and on females that miss out on mating. Importantly, there are also potential population-level effects because, as is the case with most sexually deceived pollinators, *L. excelsa* are haplodiploid insects. In haplodiploid species, eggs fertilised with sperm typically become daughters, whereas unfertilised eggs become sons (Godfray, 1990). Therefore, the sex ratio and the overall population dynamics of haplodiploid species likely depends on female sperm management, and therefore the sperm stock of the males she encounters. When orchid sexual deception results in sperm wastage, this could have multi-level impacts on male sperm allocation behaviour, female access to sperm, and the population sex ratio. In turn, the relative availability of

male and female wasps could affect the fitness of individuals, orchid pollination rates, and finally on the sustainability and evolutionary maintenance of the orchid-pollinator sexual deception system.

The potential for sexual deception with sperm wastage to influence both wasp and orchid fitness depends upon the pollinator's ability to make or replenish sperm. In male insects, the testes release mature sperm into seminal vesicles (Bressac, Damiens and Chevrier, 2008). From the seminal vesicles, males deliver sperm in spermatophores. Because of this, males can often vary their ejaculates with different environmental cues (e.g. female quality or reproductive status; or availability of females; Parker and Pizzari, 2010). Some insects can replenish their sperm throughout their life, but others cannot. For example, in many parasitoid wasps, which are commonly pollinators of sexually deceptive orchids, the spermatozoa are produced in the testes during late larval and pupal stages only (Boivin, Jacob and Damiens, 2005). Adults then emerge with their full sperm complement and will not produce any more in their lifetime (pro spermatogenic). In other insect species, males may become temporarily sperm limited, but can replenish their stocks to varying degrees (synspermatogenic). Synspermatogeny is particularly common when males encounter females over a long period, whereas a male of a

prospermatogenic species usually only encounters females early and during a short period of his life (Boivin et al., 2005; David Damiens and Boivin, 2006). Of the few studied parasitoid and solitary wasps, most are prospermatogenic (Boulton, Collins and Shuker, 2015), but the spermatogeny of *L. excelsa* is unknown. If these wasps are prospermatogenic, mating with an orchid may drastically change the sperm availability of the populations they target, and hence their evolutionary trajectory. Alternatively, males may produce sperm during their adult lifespan, and not face high costs of sperm limitation.

Research is increasingly finding that the energetic costs of sperm production are not trivial (Hayward and Gillooly, 2011; Olsson, Madsen and Shine, 1997). While sperm production is not usually as expensive as female production of ova, spermatogenesis is costly for several types of insects (e.g. Blattodea, Coleoptera, Diptera and Hymenoptera; Godwin et al., 2017; Zajitschek et al., 2019; Wagner and Bakare, 2017; Bunning et al., 2015; Nina Wedell, Gage and Parker, 2002; Boivin, 2013). Patterns of sperm production and use are particularly interesting for parasitoid insects because they are likely to be instrumental in the evolution of sexually deceptive pollination systems. Spermatogeny, however, is poorly studied in parasitoid wasps that commonly pollinate sexually deceptive orchids,

and very little is known about sperm production in ichneumon wasps, which pollinate *Cryptostylis* orchids (see Boivin, Jacob and Damiens, 2005).

Here we investigate sperm use and allocation for a sexually deceived ichneumonid wasp in populations with and without ongoing contact with orchids. Comparison of the wasp with and without selection imposed by orchids is possible because the pollinator, *L. excelsa*, has a cosmopolitan distribution, whereas the *Cryptostylis* orchids occur in smaller, discrete patches within the pollinator's range. We examine the mating potential of deceived pollinators by comparing the sperm stock and ejaculate size of males that have, or have not, been permitted to mate with *Cryptostylis* orchids in the field. If male orchid pollinators do become sperm limited, we predict limitation of sperm throughout a males' mating history. As proxies for this, we have three predictions for male sperm stock. First, males who have recently mated with an orchid (and therefore have had at least one known mating event) would have a smaller sperm stock than males who have not. Second, if males are prospermatogenic, we expect that males from sites with orchids would have smaller sperm stocks than males from sites without orchids, as they have a higher chance of depleting their stock through encountering both mates or orchids. Finally, males caught later in

the day have less sperm, as they may have had several mating events in one day. Additionally, if sperm production comes at a cost to *L. excelsa*, we would expect them to vary ejaculate size in line with optimal allocation (Abe and Kamimura, 2015; Reinhold, Kurtz and Engqvist, 2002) to mate with larger numbers of mates (either real or deceptive). We expect that males from sites without natural populations of orchids will have larger ejaculates than males from sites with natural populations of orchids.

However, we include the caveat that differences in the ejaculate size at these sites may arise from either males' experience with orchids, or males perceiving more mates in their habitat. This study does not determine the source of the difference here, as we cannot follow a focal male.

4.2 Materials and methods

Field trials

Between December and February 2016-2018, we recorded pollinator behaviour in 15-minute field trials following the methods of Bower and Brown (1997). At one experimental trial, an unpollinated flower of either *C. subulata* or *C. erecta* was exposed as bait in the field, in a mesh cage, until a single pollinator visited. We used a mesh cage to prevent more than one male from mating with the orchid bait at a time. Experiments took

place between 6:00 and 14:00h to coincide with the period of maximum activity for this wasp (Tomlinson and Phillips, 2012). We carried out these experiments at six different sites, three sites with natural populations of orchids and three sites without natural populations of orchids (chapter 2, S. Fig. 3). Sites were at least 2km apart, exceeding the dispersal range of *L. excelsa* (maximum 600m; Weinstein, Davis, Menz, Dixon and Phillips, 2016). We classified these sites into groups “with orchids” or “without orchids”; sites were matched as per the methods laid out in chapter 2 (S. Table 4).

At all sites, we tested for differences in sperm stock by comparing male *L. excelsa* that have had an unknown number of mating events with males that have had at least one known mating event by either preventing a male from mating with a focal orchid bait (n matings) or allowing him to mate with a focal orchid bait ($n + 1$ matings). We randomly assigned males to these treatments before commencement of each trial. Prevented males were caught outside of the mesh cage as they approached the orchid, while those allowed to mate with the orchid were permitted to enter the cage and continue with their typical behaviour - landing on the orchid, mating vigorously, sometimes ejaculating and/or self-grooming, then

leaving the orchid and flying up into the mesh cage, where they were caught.

If we did not attract pollinators after two trials, we removed the orchid and began a new trial 5 metres away. We used orchid flowers within 48 hours of collection and, where possible, re-used flowers used to lure wasps in the “prevented” treatment.

Sperm counts

Sperm stock in seminal vesicles was assessed for males in both treatments following the methods of Bressac et al.(2008). After capture we quickly froze and dissected males. We removed their seminal vesicles and placed them on a slide with saline (128.3nM NaCl*137mM in phosphate buffer pH 7.2) and 0.3% triton solution to avoid flattening. We photographed the seminal vesicles under the microscope and measured their area using ImageJ. They were then broken open with an entomological pin and gently stirred to disperse the sperm, fixed with ethanol, then left to dry at ambient temperature. We subsequently stained these slides for 15 minutes with DAPI (300nM in PBS). We estimated the number of spermatozoa using a fluorescence microscope. Spermatozoa counts were estimated by counting precisely ten spermatozoa, estimating the area of those ten

sperm, and multiplying that by the area of the full sperm mass to obtain the total number of cells.

We estimated ejaculate size in a similar way. After a wasp was permitted to mate with an orchid ($n + 1$), the flower was examined with a hand lens for spermatophores while still in the field. If a spermatophore was visible, it was immediately removed from the flower with forceps and placed on a slide. In situ, the spermatophore was broken on the slide with forceps and treated as per the above methods, then later stained and counted.

For some observations it was not possible to estimate the number of spermatozoa: the sperm mass was not spread thoroughly before drying, and flagella stuck together such that individual sperm cells could not be isolated and counted. These observations ($n = 39$) were removed from analyses, leaving sperm stock information available for 85 individuals.

Statistical analyses

We constructed two generalised linear models to determine the effects of mating events and exposure to orchids on sperm stock using the core stats package in R. We were interested in whether (model 1) current sperm stock or (model 2) the proportion of ‘sperm limited’ males depends on whether males have had $n + 1$ matings or not, live with orchids or the

time of capture. We defined ‘sperm limited’ males as those that had a sperm stock lower than the difference in average sperm stock before mating and after mating (30,000 sperm per mm² of seminal vesicle). This assumes that any male below that difference is sperm depleted because they will not be capable of losing the average number of sperm in their next mating event.

In addition, for males in the $n + 1$ treatment that ejaculated, we (model 3) explored the potential for males to vary their ejaculates by comparing ejaculate size (sperm count of ejaculate) to estimated total sperm stock, their prior exposure to orchids (based on whether the males were caught from sites with or without wild orchids present), or the time of day males were caught using a generalised linear model.

For analysis, all raw sperm counts were divided by seminal vesicle area as sperm number varies with animal size (Bangham, Chapman and Partridge, 2002; Schlüns, Schlüns, Praagh and Moritz, 2003; Wiernasz, Sater, Abell and Cole, 2001). Where we compared ejaculate size to total sperm stock in model 3, we estimated total sperm stock by adding ejaculate sperm count to the sperm count obtained from the same male’s seminal vesicles.

In addition to our variables of interest ($n + 1$ matings or n matings; living with or without orchids; time of capture), initial models included all possible covariates (individual site, orchid species bait, date, and year) and their interactions. Covariates that did not account for significant variation in the data (p value > 0.05) were removed in a stepwise manner to create the most parsimonious model for each question (removed only if models were significantly different, detected using log-likelihood, and if AIC was reduced, Table 1). Generalised linear models were chosen over mixed models as the data did not sufficiently support random effects, and generalised linear models had an improved fit. We corrected for multiple comparisons using Benjamini-Hochberg false discovery rate procedure. None of the models initially had normal distribution, so all dependent variables were logged to meet this assumption. Final explanatory variables used in the analysis are highlighted in Table 1.

4.3 Results

Sperm stock and limitation

Males that had $n + 1$ matings had significantly less sperm than males with n matings (p value < 0.02 , Figure 2A). The quantity of sperm in prevented males was 1.8 times as high as males permitted to mate (mean

of 40,674.67 versus 72,890.62 sperm per mm² of seminal vesicle; mean of all males 56,972.15 sperm per mm² of seminal vesicle). There was no relationship between the time of day males were caught and their sperm stock in their seminal vesicles. Similarly, sperm stock was not significantly different between males from sites with natural populations of orchids and males from sites without (post-hoc power analysis: 54.1%, Figure 2A).

The proportion of males that were sperm limited was significantly larger in the $n + 1$ matings treatment (45%) than the n matings treatment (23%; p value < 0.05 , Figure 2A). The proportion of sperm limited males did not depend on whether males were from sites with or without natural populations of orchids or the time of day that the males were caught (Figure 2B). 34% of all captured males matched our definition of ‘sperm limited’ (sites with orchids: 42%; sites without orchids: 35%).

Ejaculate size

Ejaculate size of males from sites with natural populations of orchids was smaller than that of males from sites without natural populations of orchids (p value < 0.03 , Figure 3 A). Males’ ejaculate size had a weak, significant, positive correlation with total estimated sperm stock (p value < 0.05 , Figure 3 B), but the interaction term between orchid presence and

sperm stock was not significant. Ejaculate size did not vary significantly with the time of day males were caught, but did have a weak, negative correlation. Ejaculate size varied from 5 to 25% of males' total sperm count (mean of 8% at sites with orchids and 13% at sites without orchids).

4.4 Discussion

The sperm stock of *L. excelsa* males with one known mating event with a sexually deceptive orchid ($n + 1$ matings) was significantly less than males with fewer matings (n matings). It did not decrease, however, with the time of day collected as we would expect if males depleted their sperm stock over the course of several mating events in a day. This suggests that this species may be at least moderately synspermatogenic according to the spermatogeny index developed by Boivin et al. (2005). This index ranges from 0 in prospermatogenic species (complete, limited sperm stock at emergence) to 1 in synspermatogenic species (continuous sperm production, and only temporary sperm limitation). Similarly, sperm stock did not decrease with the presence or absence of natural populations of orchids in their habitat. If we assume that males experience more mating events at sites with orchids, this suggests that in response to an increased number of mates (orchids or females) males may either increase investment

in sperm stock (so have higher initial sperm counts and despite more mating events, still match males that have had fewer mates) or replenish sperm quickly. However, the power of this analysis is only small (54%) so a future study with a larger sample size would better verify this claim.

Additionally, this finding relies on sites being precisely matched. In this study, I aimed to make sites as comparable as possible. However, because of the abundance of *Cryptostylis* orchids across Sydney, sites with orchids were sports fields contiguous to native bush, whereas sites without orchids were sports fields contiguous to planted areas. These differences in sites might drive differences in pollinator behaviour and morphology: due to host availability, food availability or searching area. Of course, exactly matching sites is difficult in ecological studies. Hence, future work might explore sperm availability and use in a lab-reared population of ichneumonid wasps -holding any differences in environment constant. This work could simulate sexual deception by allowing males to mate with females, but preventing true copulation via a barrier (e.g. grease paper used to prevent male stick insects from depositing sperm; Burke and Bonduriansky, 2019).

The mean sperm count for all males (mated or unmated) was approximately 57,000 sperm per mm² of seminal vesicles (mean raw sperm count is ~27,000 sperm). Other similarly-sized wasp species are estimated to have around 30,000 sperm (raw count; Adult male *Ancistrocerus antilope*, Family: Vespidae; Bushrow, Fuller, Cowan, and Byrd, 2006).

Unfortunately, many studies that record sperm in wasps do not account for variation in animal size (through animal length, or some morphology related to animal size such as testes or seminal vesicles). This is crucial if we want to compare sperm stock across species or within a species that has a large range of body size, as sperm counts have been shown to vary with animal size (Bangham et al., 2002; Schlüns et al., 2003; Wiernasz et al., 2001). Although, sperm count can also be influenced by an animal's previous mating history and age (Bressac, Khanh and Chevrier, 2009; D. Damiens, Bressac and Chevrier, 2003)

Moreover, we found that a significantly larger proportion of males were sperm limited in the $n + 1$ treatment than the n treatment. A large proportion of the males we captured had low sperm stocks (Figure 2); at least 20% of all male *L. excelsa* we captured had less than ~15,000 sperm/mm² (versus a population mean of ~50,000 sperm/mm² seminal vesicle) and 13% of males had essentially limited sperm stocks at time of

capture ($\sim 5,000$ vs a population mean of $\sim 50,000$ sperm/mm² seminal vesicle). These low sperm stocks indicate that males of this species may at least become temporarily sperm limited (moderately synspermatogenic), a common occurrence in reasonably long-lived species. Temporary sperm limitation has also been reported in the single available study of sperm production in an Ichneumonid wasp, *Diadromus pulchelles* (Boivin et al., 2005; Chauvin, El Agoze, Hamon and Huignard, 1988).

We found that *L. excelsa* males likely vary their ejaculate size depending on their current sperm stock. This suggests that *L. excelsa* males transfer more sperm when they have more at their disposal, as per the strategic ejaculation hypothesis (Simmons and Siva-Jothy, 1998). Ejaculate size varied from 5 to 25% of males' total sperm count (mean of 8% at sites with orchids and 13% at sites without orchids). Therefore, we may expect that males could mate about ten times in rapid succession before becoming sperm limited, even if limitation is only temporarily. At sites with orchids, male ejaculates had significantly less sperm than at sites without orchids. This suggests that males can strategically allocate their sperm. This could occur when males cannot distinguish between orchids and real females. Males may perceive orchids as "low-quality females" and therefore transfer less sperm. Alternatively, males may allocate less sperm per mating to

optimise the total number of matings they can obtain: a common response to a female-biased sex ratio (Abe and Kamimura, 2015; Allen, Barry, Holwell and Herberstein, 2011; Parker and Pizzari, 2010; Wedell and Cook, 1999). Alternatively, the smaller and less sperm-rich ejaculates we observed may arise via learned avoidance or adaptation, with selection driven by the costs of sperm wastage. Under this scenario, selection may act on males to learn to avoid orchids or evolve traits that help them to do so, such as reducing their ejaculate size when mating with an orchid. This difference in ejaculate size is unlikely to result from simple sperm limitation of males at sites with orchids because we found that males from all sites had similar seminal vesicle sperm stock.

While this is a good first step to assess sperm stock in a wasp that is difficult to rear in the laboratory, successive mating of males with females, if possible, (e.g. Boivin et al., 2005) would be a better way to measure an individual's tendency to become sperm limited. However, there are very few species for which data on spermatogeny is available. For Ichneumonids like *L. excelsa*, and closely related Braconidae, three species have been investigated, and they all appear to become at least temporarily sperm limited within 5 – 20 mating events (David Damiens and Boivin, 2006; B. King, 2000; Nadel and Luck, 1985), although they may be able to replenish

their stock over a few days. Female *L. excelsa* are likely monandrous, as for most solitary parasitoids (Quicke, 2014). The ancestral sex determination system in Hymenoptera is arrhenotokous parthenogenesis via complementary sex determination (Heimpel and de Boer, 2008). Through this mechanism, diploid females develop from fertilised eggs and haploid males from unfertilised eggs. Diploid males may also arise from complementary sex determination, however they are most often non-viable or infertile (Harpur, Sobhani and Zayed, 2013; Winkert, de Oliveira and Faria, 2019). Therefore, female *L. excelsa* likely rely on successful sperm transfer from fertile haploid males during their only mating event to produce daughters, although they can produce viable sons regardless of whether they mate. If they mate with a sperm-limited male (in our study, this is approximately 21% males at sites with orchids) and do not receive a full complement of sperm, they could be considered ‘pseudo-virgin’ – never having received enough viable sperm in their lifetime to produce daughters and constrained to produce only sons.

If females suffer from sperm limitation, this can alter the costs and benefits of multiple mating and females may accept more than one mating (Boulton and Shuker, 2015). However, this is unlikely as parasitoid females cannot detect male sperm status and often do not increase mating

frequency if sperm limited (Abe, 2019; King and Fischer, 2010; King and Miller, 2018; Steiner, Henrich and Ruther, 2008). Analysis of sperm viability in ejaculates would help estimate the proportion of viable sperm a female might expect to receive, even in a mostly limited male. It may also be useful to compare viability of sperm transferred to orchids versus sperm transferred to true females, to see whether males vary their ejaculate quality, as well as quantity, depending on perceived mate quality.

Additionally, it would be interesting to compare female mating frequency in *L. excelsa* between sites with and without orchids to determine whether they change behaviour in response to orchid-induced sperm limitation.

Quantifying the costs of sexual deceit is crucial, particularly as these relationships were previously assumed to be benign and non-coevolutionary (Schiestl, 2005; but see Wong and Schiestl, 2002). *Cryptostylis* deception offers an exciting avenue of research for sexual deception generally, and a broader understanding of the maintenance of asymmetric relationships.

Understanding whether sperm is a limiting resource is important for exploited populations such as *L. excelsa*, who unwittingly waste sperm on something other than reproduction. This phenomenon is not, however, restricted to sexually deceptive orchids. For instance, while the Amazon molly, *Poecilia formosa*, requires the sperm of closely related heterospecific

males to reproduce – it does not use their sperm (Hubbs and Hubbs, 1932). Raising the question, is sperm availability or production a limiting factor for unwitting *Poecilia* sperm donors? The extent of these costs may also vary depending on a species' life history. For example, the specifics of wasps' mating systems may make them uniquely suited to sexual exploitation by orchids. Diploid females (e.g. flies and beetles) cannot reproduce without sperm and thus may be particularly vulnerable and unsustainable targets for exploitation. Perhaps correspondingly, they are less common pollinators of sexually deceptive orchids (Gaskett, 2011; Van Der Cingel, 2001). Conversely, haplodiploid insects (e.g. parasitoid wasps such as *L. excelsa*) can reproduce with or without sperm, although females that miss out on sperm likely produce only sons (Heimpel and de Boer, 2008). This may well explain the vast preponderance of haplodiploid wasp and bee pollinators for sexually deceptive orchids worldwide: sperm- or mate-limited females may be constrained to produce only sons, while females with sperm can choose between son or daughter production. If a large proportion of females are constrained to produce sons, this may create a stable male-biased sex ratio (Lehtonen and Schwanz, 2018; Rautiala, Helanterä and Puurtinen, 2017) which allows for enough males in the exploited population to share between orchids and female insects.

It follows that sperm limitation could impact an exploited species' persistence, and (in the case of haplodiploids) sex ratio, with flow-on effects for population dynamics, and interactions with other species such as sexually deceptive orchids (Gaskett et al., 2008). This idea has until now lacked empirical support. Our results demonstrate that insects fooled into pollinating sexually deceptive orchids may indeed have limited sperm and vary sperm transfer in response to that. Despite previously held beliefs that deceptive flowers should evolve to have minimal impact on their pollinators, it appears that these orchids not only affect individual pollinator behaviour but may influence the population dynamics of their pollinators. This research opens new opportunities for understanding co-evolutionary processes between deceivers and their dupes.

4.5 Tables and figures



Figure 1 *Cryptostylis subulata* and its pollinator, the ichneumonid ‘Orchid dupe wasp’ (*Lissopimpla excelsa*) post copulation. Note the orchid’s yellow wishbone-shaped pollinia attached to the abdomen of *L. excelsa*. Photograph by A L Brunton Martin.

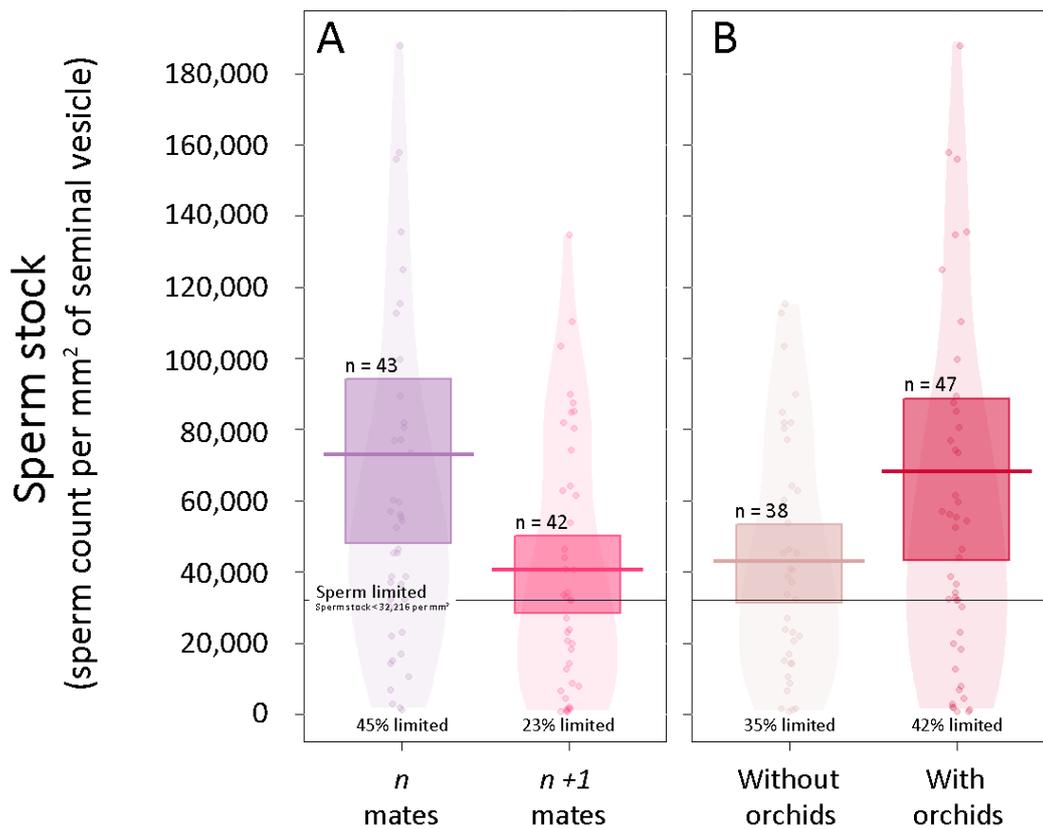


Figure 2: *Lissopimpla excelsa* (Ichneumonidae) males that (A) had mated $n + 1$ times (with an orchid in our trials) were proportionally more sperm limited than males that had mated n times (, i.e. prevented from mating with an orchid in our trials; < 5000 sperm – indicated by line and text below dotted line with predicted percent of limited males; binomial GLM p value < 0.05). In addition, males that have mated $n + 1$ times have significantly less (GLM p value < 0.05) sperm stock overall (sperm count / seminal vesicle area) than males that have mated n times; (B) came from sites without natural populations of orchids did not have significantly larger sperm stock than males that came from sites without natural populations of orchids, nor were they more sperm limited (power analysis 54%). Note, here we display untransformed counts – models used logged values to meet assumptions of normality. Points indicate raw data, with a smoothed density curve showing data distribution. Box indicates median and 95% confidence interval. Sample sizes are displayed on top of boxes.

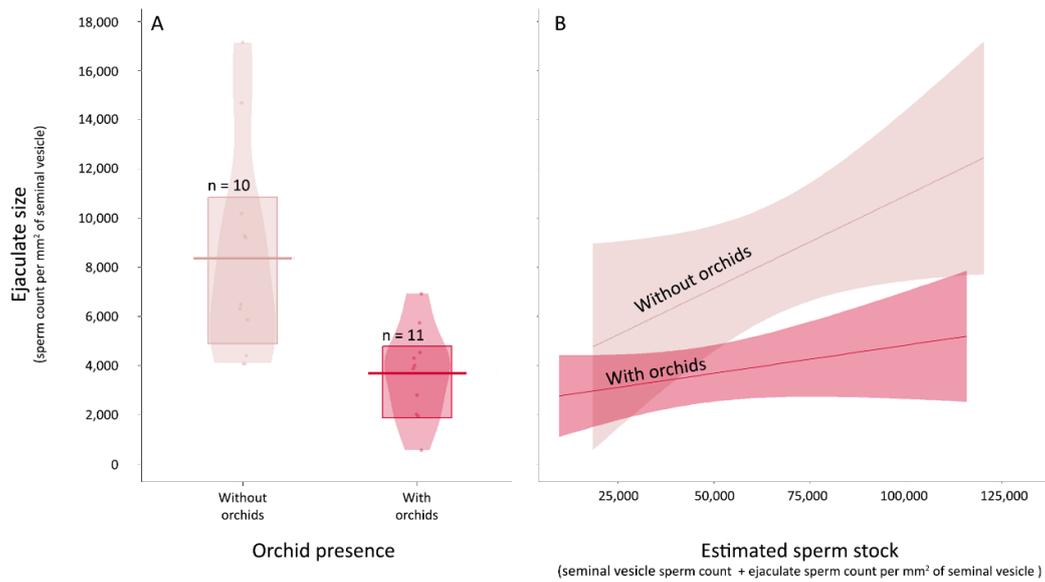


Figure 3: (A) *Lissopimpla excelsa* males that co-occur with orchids deposit significantly less sperm than males that do not co-occur with orchids (GLM p value < 0.05). (B) Males from sites with and without orchids increase the number of sperm in their ejaculates with increasing total sperm stock (GLM p value < 0.05). Note, here we display untransformed counts – models used logged values to meet assumptions of normality. Points indicate raw data, with a smoothed density curve showing data distribution. Box indicates median and 95% confidence interval. Sample sizes are displayed on top of boxes.

Table 1: Summary of statistical models used for analysis.

Model no.	Description	Initial AIC (all variables)	Final AIC (parsimonious)	Dropped covariates	Final predictive variables
1	Generalized linear regression	308	161	Individual sites, date of collection and species of orchid bait	Time of day, orchid exposure, mating treatment, year
2	Logistic regression	111	105	Individual sites, date of collection, year and species of orchid bait	Time of day, orchid exposure, mating treatment
3	Generalized linear regression	11	7	Individual sites, date of collection, year and species of orchid bait	Time of day, orchid exposure, mating treatment

Chapter 5: Haplodiploidy buffers the costs of extreme sexual deceit.

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Sciences*

5.1 Introduction

Male behaviour is seldom considered a driver of population dynamics and, typically, population dynamic models assume that males do not limit female reproduction (Caswell, 2001; Engen, Lande and Saether, 2003; Hamilton, 1967; Rankin and Kokko, 2007). However, there is considerable evidence that male presence or behaviour impacts females. By sharing or guarding resources, males change resources available to females (Clutton-Brock, Coulson, Milner-Gulland, Thomson and Armstrong, 2002; Dreiss, Cote, Richard, Federici and Clobert, 2010), and may also kill or injure females (Galliard, Fitze, Ferrière and Clobert, 2005; Kamimura, 2012; Kokko and Rankin, 2006; Réale, Boussès and Chapuis, 1996; Rice, 2000). Importantly, male traits can change females' access to sperm: for example, males may always have plentiful sperm stocks, or could be sperm depleted,

sperm limited, or strategically allocate their sperm (Dyson and Hurst, 2004; Parker and Pizzari, 2010; Reinhold, Kurtz and Engqvist, 2002; Wedell, Gage and Parker, 2002).

Sperm can be limited in many circumstances. Male killers such as *Wolbachia* or *Spiroplasma* influence adult sex ratios by decreasing the number of males born, which limits the availability of mates for females (Hayashi, Watanabe, Yukuhiro, Nomura and Kageyama, 2016; Pontieri, Schmidt, Singh, Pedersen and Linksvayer, 2017). Male presence does not always guarantee sperm availability either: for example, the parasitic fungus *Massospora cicadina* that infects *Magicicada* cicadas destroys their genitalia and changes male behaviour. Healthy males attempt to copulate with, and waste sperm on, infected males (Cooley, Marshall and Hill, 2018). Similarly, in sexually deceptive orchids, flowers attract pollinators by imitating female insect mating signals, especially their pheromones and morphology (Bohman, Phillips, Flematti, Barrow and Peakall, 2017; Bohman, Weinstein, Phillips, Peakall and Flematti, 2019; Gaskett, 2012; Gaskett and Herberstein, 2009; Peakall and Handel, 2013; Schiestl et al., 2003; Spaethe, Moser and Paulus, 2007). The male parasitoid wasp, *Lissopimpla excelsa*, is deceived in this way by *Cryptostylis* orchids, and

frequently ejaculates onto the flowers (Gaskett, Winnick and Herberstein, 2008).

For many insects, sperm is not cheap. The energetic demands of sperm production can result in a reduced body mass, shortened life span, or limited total sperm production over a lifetime (Boivin, Jacob and Damiens, 2005; Wedell et al., 2002). The majority of insects fooled by sexually deceptive orchids are solitary bees and parasitoid wasps, where sperm is generally limited (Boulton, Collins and Shuker, 2015). Male parasitoid wasps can become completely sperm depleted after between 5 and 20 mating events (Damiens and Boivin, 2006; King, 2000; Nadel and Luck, 1985). Though ejaculation is confirmed in only one sexually deceptive system (*Cryptostylis* orchids ; Brunton Martin, Gaskett and O’Hanlon, 2020; Gaskett et al., 2008) and suggested in another (*Lepanthes* orchids; Blanco and Barboza, 2005), all sexually deceptive orchids interfere with pollinator mate-searching and copulation behaviour, with associated costs. Most pollinators of sexually deceptive plants have mating systems involving scramble competition (Boulton et al., 2015; Kai-Fong, Brunton Martin and Gaskett, 2019), in which there is intense male-male competition to be the first to find and mate with a female. Thus, any search time that males lose in pursuit of an orchid is a potential

opportunity cost that might result in missed mating opportunities. Missed matings can translate to reduced mating rates for females who live near orchids, since males cannot discriminate between, and often prefer, orchid odours to those emitted by females (Jager and Ellis, 2014; Peakall, 1990; Wong, Salzman and Schiestl, 2004; Wong and Schiestl, 2002). For some species, missed matings may also result in missing out on food gifts from males and transport to oviposition sites (Wong and Schiestl, 2002). There is also the possibility that when a male finds a female he is already sperm depleted due to previous encounters with orchids. Solitary parasitoid females are only receptive to a few mating events (they mate monandrously or at most twice (Ridley, 1993; Sandanayaka, Chhagan, Page-Weir, Silva and Charles, 2011)). Thus, if their few mating events occur with sperm depleted males, insufficient sperm may limit their lifetime reproductive success.

Given the mating and reproductive traits of the insects fooled into sexual deception, orchid-imposed mate or sperm limitation likely impacts the population dynamics of orchid pollinators. In general, models that consider strictly monogamous populations suggest that population dynamics are susceptible to mate limitation (Bessa-Gomes, Legendre and Clobert, 2004). In polygynous population models, however, mate limitation typically has

little effect on population growth, since a small number of males are assumed able to fertilise many females (Rankin and Kokko, 2007).

However, as no male can have infinite sperm production capability, sperm limitation remains a possibility under polygynous mating systems.

If orchids interfere in pollinator reproduction by imposing sperm limitation (either through depleting males or preventing males from encountering females in their lifetime), I would expect flow-on effects for pollinator population dynamics and, hence, limit the persistence of both the pollinator and deceptive orchid. Nevertheless, sexual deception is a surprisingly common pollination system, reported for over 400 species (Gaskett, 2011). A key factor that might mitigate the costs of deception is haplodiploidy. Approximately 95% of known pollinator species of sexually deceptive orchids are haplodiploid, and in particularly solitary bees and wasps (Kai-Fong et al., 2019). Usually, under haplodiploidy, eggs fertilised with sperm typically become daughters, while unfertilised eggs become sons (Godfray, 1990). While diploid males may also arise from fertilised eggs in this system, they are most often inviable or infertile (Heimpel and de Boer, 2008).

Here, I model the effect of orchid deceit and consequent sperm wastage on female reproductive success and population persistence in a population of

polyandrous males and monandrous females, as would be typical for insects involved in sexually deceptive pollination systems. Given the prevalence of haplodiploidy in sexually deceptive systems (Chapter 1; Gaskett et al., 2008; Kai-Fong et al., 2019), I specifically evaluate whether the population dynamic consequences of sperm limitation differ between haplodiploid and diploid pollinator species. There are two reasons why haplodiploidy might buffer against the adverse population-level effects of sexual deception.

First, a haplodiploid female, when deprived of sperm, is still able to reproduce – although all her offspring will be male (Normark, 2003; Rautiala, Helanterä and Puurtinen, 2017). Second, haplodiploid females (if fertilised) can facultatively adjust their offspring sex ratio by controlling the proportion of their eggs that are fertilized (Burton-Chellew et al., 2008). In other words, a population of mothers of haplodiploid species comprise virgin mothers that produce only sons, and mated mothers who specialize in daughter production, but may also produce some sons.

Together, these would increase the production of pollinators; this should provide enough males to fertilise females and help maintain the population despite some male mating effort being wasted on orchids (e.g. Fig. 1). I include this feedback loop on sex ratio dynamics in this model, and test for the population persistence of haplodiploids against that of diploids that

lack virgin reproduction, and the consequent overproduction of sons and sex ratio biases.

5.2 The model

Background

The haplodiploid version of this model is based on the life cycle of ichneumonid wasps, as per the pollinator fooled into ejaculating on *Cryptostylis* orchids. In this model, males emerge before monandrous females (protandry) (Quicke, 2014). Females stop investing in pheromone production after mating, irrespective of whether they have received a sufficient amount of sperm (Ridley, 1993). Under ideal laboratory conditions, female ichneumonids typically produce between one and four eggs daily. As the rate of egg production is phylogenetically constrained across parasitoid groups (Jervis, Moe and Heimpel, 2012; Price, 2003), I use these estimates in the model. Egg production lasts throughout a female's lifespan, and most females will begin oviposition after emerging, regardless of whether they have mated (Ode, Antolin and Strand, 1997).

In this model, as for parasitoid wasps in general, males have a finite capacity, defined as the number of times each male can mate before being classified as sperm depleted. Sperm depleted males will continue to mate,

but will not transfer any sperm to females (Boivin et al., 2005).

Monandrous females mating with such males become ‘pseudo-virgin’, constrained to produce only sons for life. I assume that the populations coexist with orchids long enough for sex ratio adjustments to evolve, and thus the sex ratio produced by mated females is allowed to depend on the son production by virgins and pseudo-virgins (Rautiala et al., 2017). Since I assume this is an evolutionary response, I do not assume that females can directly measure son production of others, but that females producing optimal sex ratios have been selected for.

The diploid version of the model uses the same life cycle, except for the consequences of diploidy: unmated females, or females who mated with sperm-depleted males, cannot produce offspring of either sex, and those with sperm produce offspring with a 1:1 sex ratio. I provide definitions of model variables and symbols in Table 1.

Model summary

Mating events

My model tracks the population dynamics of either a haploid or a diploid population of pollinators. I assume that there are D orchids within a unit area of habitat. Here, ‘unit’ is defined as the area required to sustain K pollinators (measured at the beginning of the season, i.e. all the recruited

offspring from the previous season). In the absence of orchids or sex ratio biases, this area will contain $K/2$ females and $K/2$ male pollinators at the beginning of the season. Note that I use the term ‘pollinator’ to refer to individuals of either sex, though only males actively contribute to pollination. My results apply regardless of the value of K since it is a parameter that scales the unit area. Consequently, there being D orchids per wasp applies across all values of K (my examples use $K = 2$).

I track the population over T_{\max} generations, where every generation occurs over a season of length S days, and each day is split into 100 time-steps (thus the total season length is $100S$ time steps). Dividing days into 100 steps is designed to have each time step short enough to represent a discretized approximation (Euler method) of differential equations, where each step can be assumed to yield maximally one encounter for each focal individual (valid across all reasonable values of male mate search efficiency). T_{\max} is chosen to be large enough ($T_{\max} = 50$) such that the population always stabilizes or goes extinct (in practice, since this model is deterministic, these happen quickly, in 20 generations or less). Generations are non-overlapping.

At the beginning of each generation (first time step of the first day of the season), males emerge. Since I need to track the mating status of the

males, I assume a male mating capacity of c matings before becoming sperm depleted. The population is initialized with a density of $M_0(t) = K/2$ males at $t = 1$, with the subscript 0 indicating the male's mating status, i.e. the number of times they have mated so far. All other $M_i(t)$ values, for $i = 1, 2, \dots, c$, are set to 0, as all males are initially in a virgin state. If a male of category c mates again, I assume him to stay in category c , since there is no need to track the precise numbers of matings for a male who is already sperm depleted. 'Matings' with orchids count towards a male's mating status, since these deplete his sperm just like matings with females do (I assume males do not 'learn' to avoid approaching or ejaculating on orchids).

Females (F) are initialized using a similar procedure, but with two differences: protandry makes them arrive later, and I need to track not only how many times females have mated (0 or 1, due to my assumption of protandry) but also whether they have received sperm (0 or 1). Note that it is possible to have mated but not have received sperm if the mating event happened with a depleted (category c) male. I, therefore, track the densities $F_{jk}(t)$, where the subscript j refers to the former aspect of female state (0 = virgin, 1 = nonvirgin), and k to the latter (0 = without sperm, 1 = with sperm). This means that $F_{01}(t)$ females cannot exist for any t ,

and I do not keep track of this variable. All other $F_{jk}(t)$ are set to zero until the emergence time point $t = 100T_P + 1$, where T_P measures the length of protandry in days. For example, if $T_P = 2$, then females emerge at $t = 201$, i.e. 200-time steps (two full days) after the male emergence time $t = 1$. At this t , I set $F_{00}(t) = K/2$ and keep the other $F_{jk}(t)$ as zero. At each time step ($t = 1$ onwards), all males search with search efficiency s . The number of encounters between a male of mating category i and a female of category 00 is

$$C_i(t) = s M_i(t) F_{00}(t) dt,$$

where dt is set to $1/100$ in accordance of my decision to split one day being into 100 time steps, and the letter C is used as these encounters are between conspecifics. Note that all $C_i(t)$ values are zero before female emergence, and that males do not mate with other categories of females than 00 (since I assume monandry). Males also mate with orchids (at all t , i.e. both before and after females have emerged), and encounter them at rate

$$H_i(t) = s M_i(t) D dt$$

where the letter H is used to denote heterospecific encounters.

The temporal dynamics is then computed, for males, as

$$M_i(t + 1) = M_i(t) - C_i(t) - H_i(t) + C_{i-1}(t) + H_{i-1}(t)$$

This formulation takes into account that males that have mated i times may stop being in this category (the terms $-C_i(t) - H_i(t)$) if they mate either with females (conspecifically) or with orchids (heterospecifically); the number of these males can also increase from the previous matedness class (category $i-1$) if such males mate, again either with females or with orchids (the last two terms). The two special categories are $i = 0$, for which I omit the last two terms (there cannot be fewer matings than 0), and $i = c$, for which I omit the two negative terms $-C_i(t) - H_i(t)$, since a sperm depleted male will never stop being sperm depleted (I assume negligible sperm replenishment; therefore males do not move on from a sperm depleted state to any other).

For females, there are three categories to keep track of: virgin (00) females, mated but without sperm (10), and mated with sperm (11). Matings with non-depleted males bring a female to a mated state, which may or may not also give her sperm:

$$F_{00}(t + 1) = F_{00}(t) - \sum_{i=0}^c C_i(t)$$

$$F_{10}(t + 1) = F_{10}(t) + C_c(t)$$

$$F_{11}(t+1) = F_{11}(t) + \sum_{i=0}^{c-1} C_i(t)$$

Reproduction

I next track the egg production of females. For practical reasons (I need to compute u , as described below), this is done for each t after the entire sequence $M_i(t)$, $F_{00}(t)$, $F_{10}(t)$ and $F_{11}(t)$ is known for the current season. I assume a delay of T_E days from female emergence to the commencement of egg production; thus egg production is checked from time $t = 100$ ($T_P + T_E$) + 1 onwards. The rules of egg production differ between diploids and haplodiploids. For diploids, only category 11 females (mated, and with sperm) can reproduce. For diploids, half of the produced eggs are male (denoted E_M), half are female (denoted E_F). Diploid egg production at time t equals

$$E_M(t) = \begin{cases} \frac{f}{2} F_{11}(t) & t \geq 100 (T_P + T_E) + 1 \\ 0 & \text{otherwise} \end{cases}$$

$$E_F(t) = \begin{cases} \frac{f}{2} F_{11}(t) & t \geq 100 (T_P + T_E) + 1 \\ 0 & \text{otherwise} \end{cases}$$

Here f is the egg-laying rate.

For haplodiploids, any female can contribute to male production, while only mated females with sperm (category 11) can produce daughters. I assume that the sex ratio, r , that the category 11 females choose has

evolved to take into account the overall level of spermlessness among females. Averaged over the entire season, the proportion of such mothers is

$$u = \frac{\sum_{t=100(T_P+T_E)+1}^{100S} F_{00}(t) + F_{10}(t)}{\sum_{t=100(T_P+T_E)+1}^{100S} F_{00}(t) + F_{10}(t) + F_{11}(t)}$$

Category 11 females then adopt a sex ratio that comes from the analysis of Rautiala et al.(2017).

$$r = \begin{cases} \frac{(1-2u)}{2(1-u)} & \text{if } u < 0.5 \\ 0 & \text{if } u \geq 0.5 \end{cases}$$

The overall egg production for the haplodiploid case takes the form

$$E_M(t) = \begin{cases} f(F_{00}(t) + F_{10}(t) + rF_{11}(t)) & t \geq 100(T_P + T_E) + 1 \\ 0 & \text{otherwise} \end{cases}$$

$$E_F(t) = \begin{cases} (1-r)fF_{11}(t) & t \geq 100(T_P + T_E) + 1 \\ 0 & \text{otherwise} \end{cases}$$

Note that the computation of r is based on females having evolved to cope with the currently valid level of spermlessness, u . At evolutionary equilibrium, which is the solution the dynamic is converging to, this can be expected to apply accurately. Before that, when u undergoes transient changes, this is best seen to be an approximation. I always report equilibrium values in the main text and figures.

In both cases (diploid and haplodiploid), the next generation is initiated with M males and F females. While the initial population densities were set to $K/2$ of either sex as explained above, the new generation of recruits is computed as

$$M = \begin{cases} \sum_t E_M(t) & \text{if } \sum_t (E_M(t) + E_F(t)) \leq K \\ \sum_t E_M(t) \frac{K}{\sum_t (E_M(t) + E_F(t))} & \text{if } \sum_t (E_M(t) + E_F(t)) > K \end{cases}$$

$$F = \begin{cases} \sum_t E_F(t) & \text{if } \sum_t (E_M(t) + E_F(t)) \leq K \\ \sum_t E_F(t) \frac{K}{\sum_t (E_M(t) + E_F(t))} & \text{if } \sum_t (E_M(t) + E_F(t)) > K \end{cases}$$

Here, the sum is applied over all t belonging to the current season (1, 2, ..., 100 for season 1, 101, 102, ..., 200 for season 2, etc.). The number of recruits is computed considering that the total population (males + females) cannot exceed K per unit area, while too low productivity means that the new generation may start with fewer than K pollinators. The recruits M and F form the M_0 and F_0 values at the appropriate t , as explained above.

5.3 Results and discussion

Haplodiploids persist where diploids do not

Every value of male capacity ($c = 1 \dots 30$) associates with a threshold density of orchids, above which the pollinator population goes extinct (Fig. 2). Unsurprisingly, as male capacity increases, populations can tolerate higher densities of orchids (all curves increase in Fig. 3). The persistence threshold is always higher for haplodiploids than for diploids, supporting my original hypothesis that haplodiploidy buffers against the negative impacts of exploitation by orchids and thus enhances the stability of the two-species system. The difference between haplodiploids and diploids arises across all examined values of male search rate (s), female emergence (T_P) or egg production (f).

Under realistic situations, where females lay an egg each day, males have several mating events in a day and females emerge later than males, the absolute difference in the persistence threshold remains small (Fig. 2 A).

Under these conditions, diploids cannot withstand an orchid density larger than 1.6 (interpretable as 1.6 orchids for every female) even if males can mate up to 30 times; for haplodiploids, the corresponding number is 1.7.

The difference between diploids and haplodiploids is more striking when

comparing orchid densities that reduce pollinator production by 50%.

Orchid densities that halve diploid daughter production cause minimal reduction (1%) in the daughter production of haplodiploids.

The superior performance of haplodiploids is associated with shifts in sex ratio (Fig. 2 B). Such shifts do not occur in diploids, who simply face extinction if the combination of male mating capacity, male search ability and orchid density do not yield enough sperm for females. At densities where diploids become extinct, haplodiploids are still able to keep some of their females fertilized (thus permitting some daughter production), aided by highly male-biased sex ratios (e.g. between 90% and 95% males at an orchid density that causes diploid extinction in Fig. 2 B) due to frequent matelessness of females at equilibrium. At even higher orchid densities, this mechanism fails for haplodiploids too, leading to extinction.

Both in diploids and in haplodiploids, population persistence in the presence of orchids is strongly impacted by the efficiency of male searching and the day females emerge (Fig. 3). Perhaps counterintuitively, high male search efficiency (panels with $s=10$ in Fig. 3) lowers population persistence (contrast with $s=1$, e.g. Fig. 3B has lower persistence than 3A, and likewise for all other pairs). However, one can explain this because high search rates combined with protandry causes there to be time when

searching males only encounter orchids, as females have not yet emerged. Highly efficient males find many orchids to mate with and are already depleted when females begin emerging, particularly so if protandry is strong (females emerge several days after males begin searching for them; in this case, high search by males makes persistence very difficult, Fig. 3D,H). If protandry is totally removed, both diploids and haplodiploids can persist up to extremely high orchid densities (Fig. 4). Thus, the best parameter values for enhanced resilience of the pollinator population combine the absence of protandry with high male mating capacity and low male searching.

The stability of *Cryptostylis* orchids and their pollinators may thus be partly explained by sperm depletion creating more pollinators (when unmated females produce only sons) – but only when targeting haplodiploids. Although this model simply derived consequences of specific (fixed) orchid densities for the pollinator and thus did not track pollination success or the consequences for orchid dynamics, the results suggest it may be advantageous for orchid deception to be so persuasive that it elicits pollinator ejaculation, and not only less extreme mate approach or pseudo-copulation behaviours. Should this lead to unmated females in the local pollinator pool, there will be more male pollinators in the next generation,

and since *Cryptostylis* is a perennial plant, the plant may enjoy elevated pollinating success during its lifespan. Indeed, simply targeting a haplodiploid pollinator (without ejaculation) may confer some benefit to orchids: diploids are typically worse pollinators than haplodiploids, although this requires formal analysis (Gaskett, 2011). Haplodiploidy also helps with persistence, should an orchid subsequently reach densities high enough to cause damage to its pollinator's population dynamics. At a density of orchids where diploids go extinct, haplodiploids can persist with a stable, male-biased sex ratio.

These findings highlight how important it is to consider males when exploring population processes and a reminder of how much it can matter to a population if females remain unmated (Rhainds, 2010). In this context, orchids may cause females to fail to achieve a successful mating – either through mating with a spermless male, or never encountering a male in their lifetime. Indeed, this model suggests female mating failure impacts populations even if sperm is not costly, simply because males could encounter orchids more frequently than females. Nevertheless, haplodiploidy might reduce the costs of missed mating opportunities for females and improve populations' chances of persistence. This might explain the prevalence of haplodiploid pollinators in sexually deceptive

interactions that I see today (Gaskett et al., 2008): relationships between sexually deceptive orchids and diploid pollinators became extinct, while those between sexually deceptive orchids and haplodiploid pollinators persisted. Indeed, it is intriguing to consider that such processes may be important for the maintenance of costly relationships in general: aspects of a species' life history may allow individuals to withstand costs of exploitation and ultimately provide population persistence.

5.4 Tables and figures

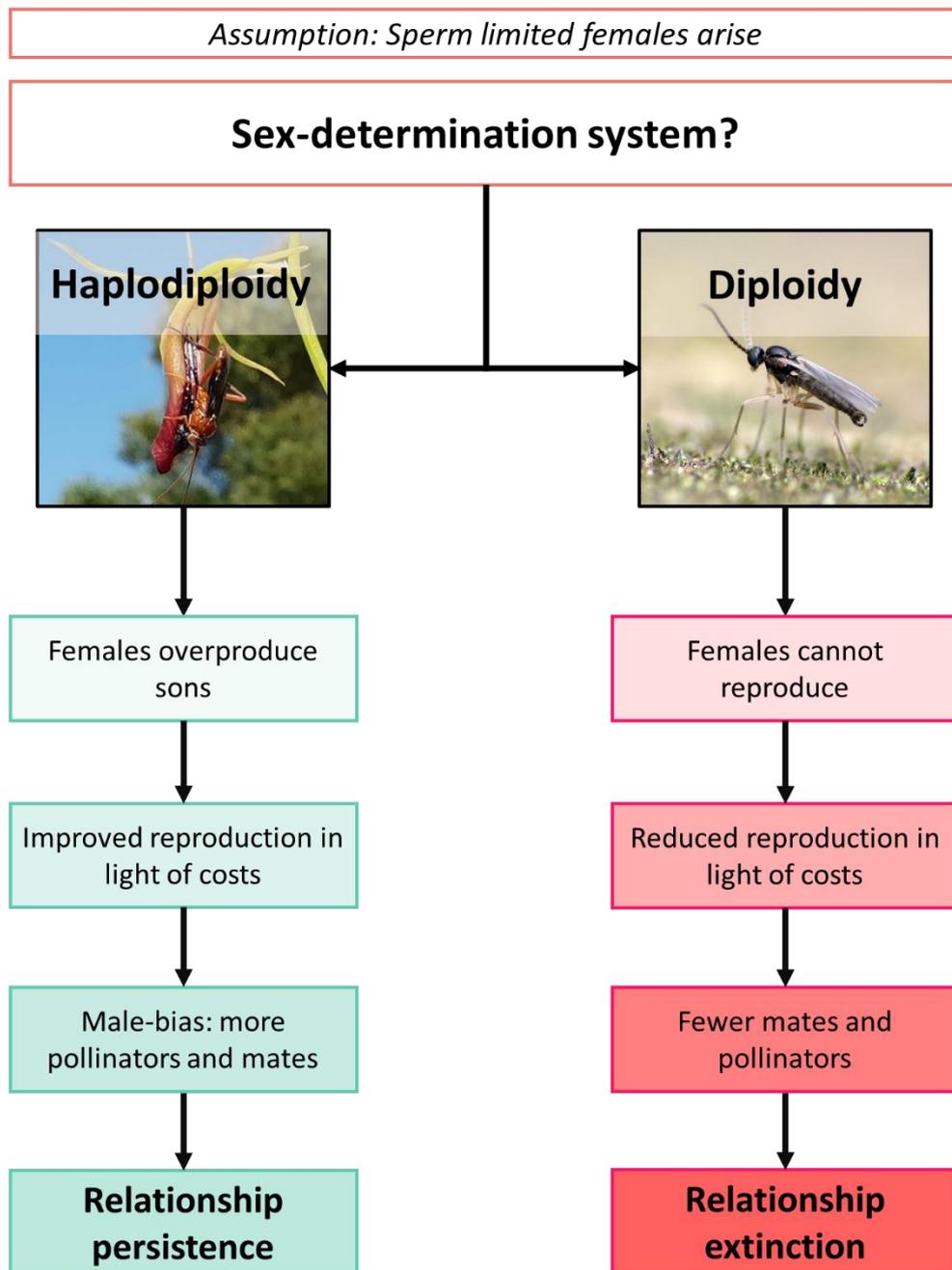


Figure 1: Reproductive strategies of sexually deceived pollinators and their hypothesized outcomes under orchid-induced sperm limitation. Images courtesy of C. Young (haplodiploid wasp, *Lissopimpla excelsa*) and K. Schulz (diploid fungus gnat, *Odontosciara nigra*).

Table 1: Definitions for model variables

Symbol	Definition
K	The number of pollinators (male and female) that can be sustained in a unit area; = 2 for all simulations
T_{\max}	The total number of generations; = 50 for all simulations.
S	Season length; = 50 for all simulations (unless stated otherwise)
c	Male mating capacity; = 30 for all simulations (unless stated otherwise)
D	Orchid density; the number of orchids in an area for every one female
s	Search efficiency
f	Egg laying rate
T_P	Length of protandry in days
T_E	Number of days since female emergence that egg production begins; = 1 (unless stated otherwise)

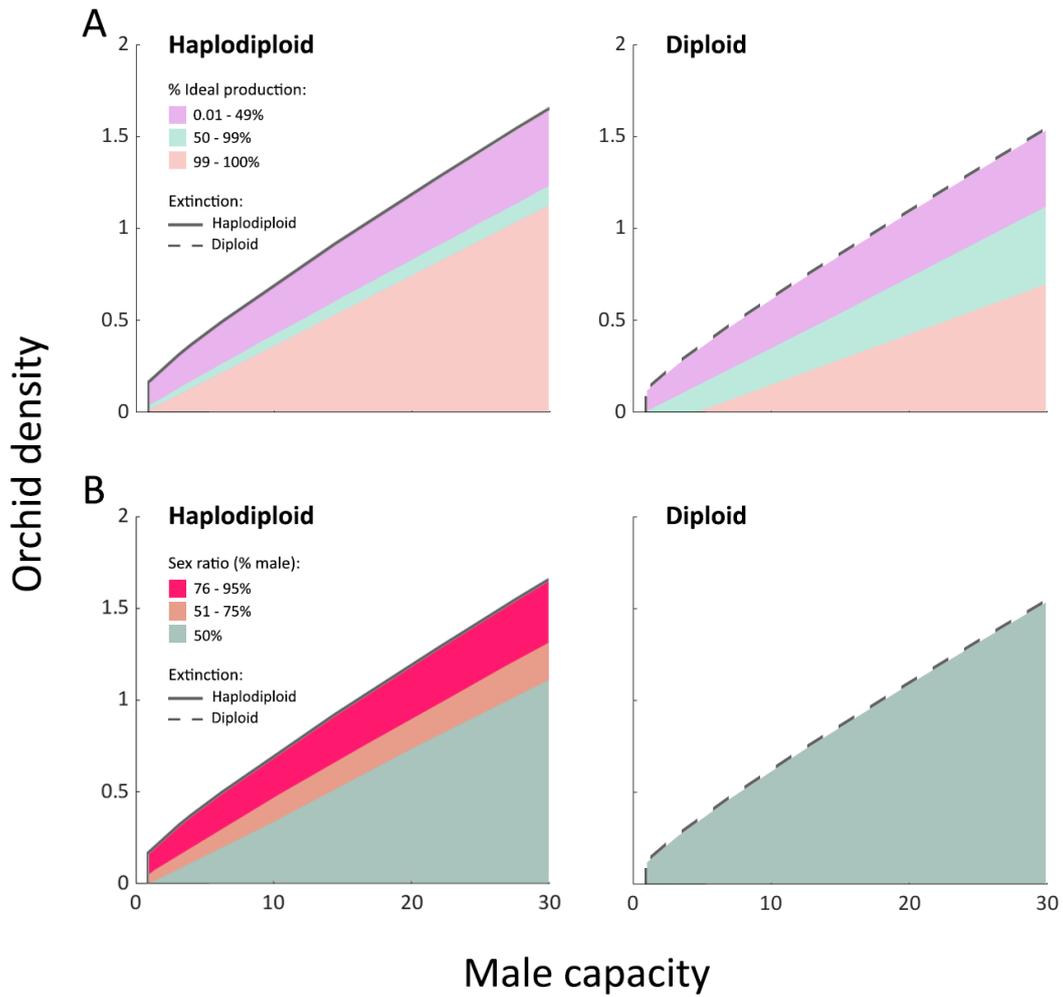


Figure 2: (A) Daughter production of haplodiploid and diploid populations. Orchid density (D) reflects the number of orchids in the environment per true female, male capacity (c) is the number of times a male can mate before he becomes sperm depleted. Colours indicate the threshold orchid density that permits 0.01, 50% or 100% of the production level of the best-case scenario to be maintained, with best-case computed within each panel as daughters produced per female when $c = 30$ and $D = 0$. Persistence thresholds given by grey lines, above these populations are extinct. (B) Sex ratio predictions. Diploid populations do not adjust sex ratios as there is no production of males caused by matelessness, sex ratio is 1:1 everywhere below the persistence threshold (grey dash line), and the population is extinct above it. For haplodiploids, sex ratios are indicated by colour. Extinction (via all offspring becoming male) is given in grey. $f = 1$; $T_P = 5$; $s = 5$.

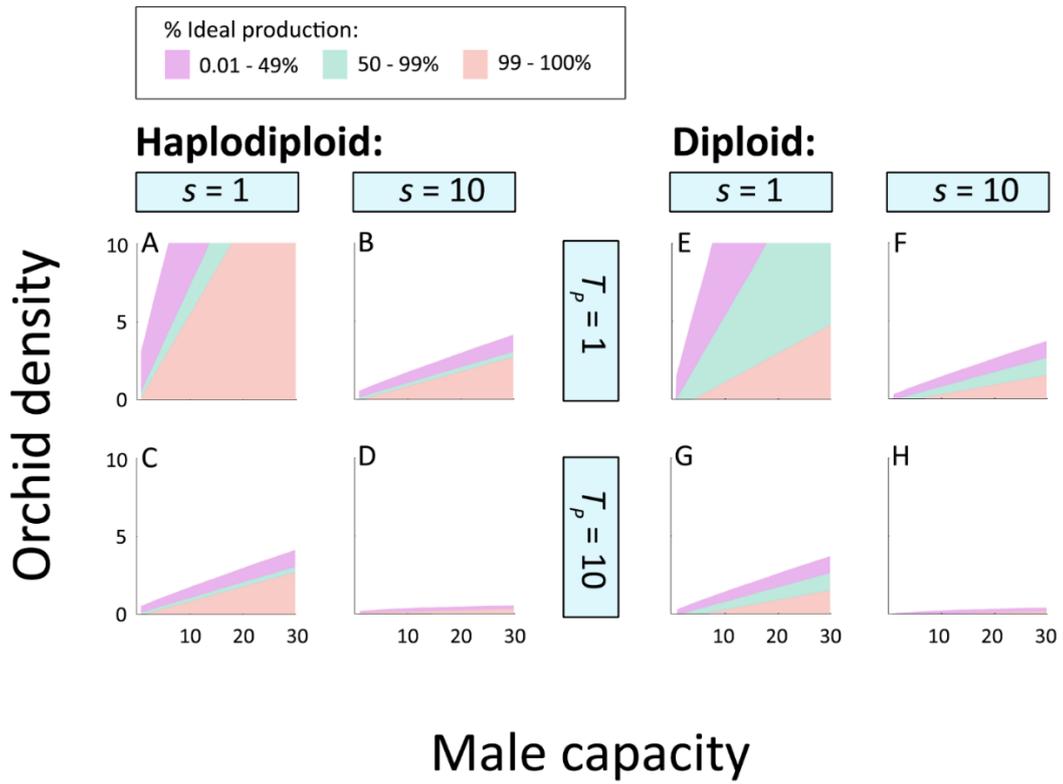


Figure 3: Daughter production of haplodiploid (left) and diploid (right) populations for decreasing levels of T_P and s , while f remains constant at 1. Orchid density (D) reflects the number of orchids in the environment per true females, male capacity (c) is the number of times a male can mate before he becomes sperm depleted. Note that all curves decrease – colours indicate the orchid density that permits 0.01, 50% or 100% of the production level of the best-case scenario to be maintained, with best-case computed within each panel as daughters produced per female when $c = 30$ and $D = 0$.

A/E: $s = 10$, $T_P = 10$; B/F: $s = 10$, $T_P = 1$; C/G: $s = 1$, $T_P = 10$; D/H: $s = 1$, $T_P = 1$

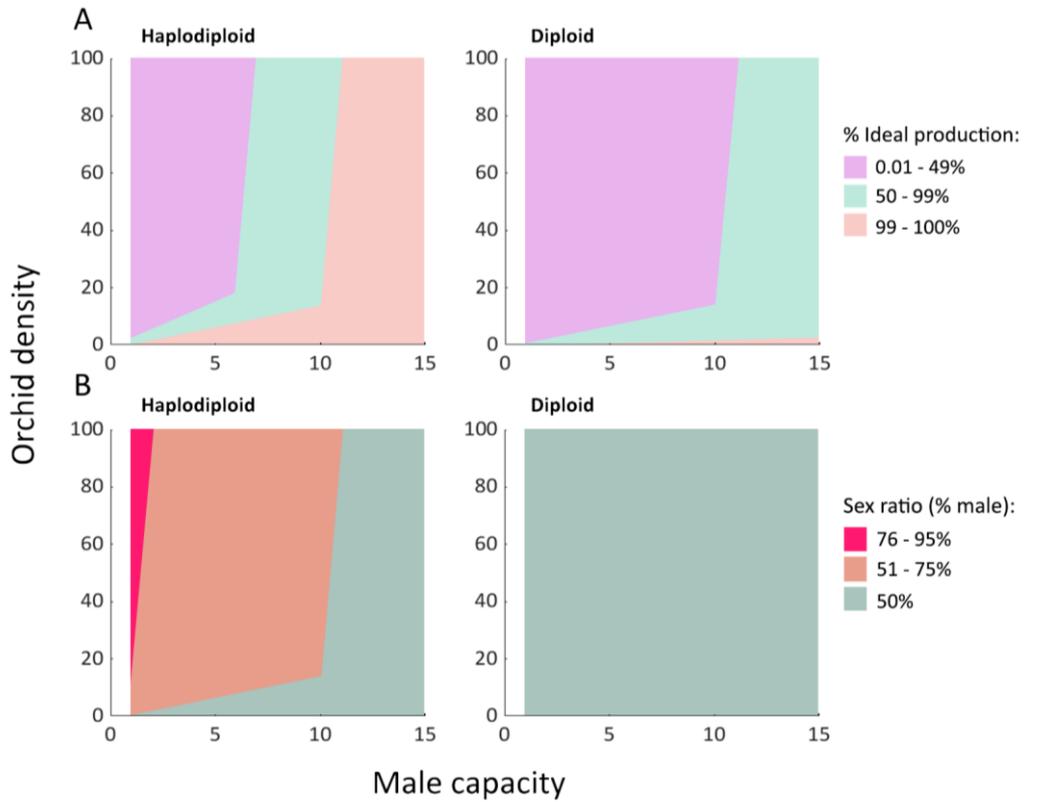


Figure 4: (A) Daughter production of haplodiploid and diploid populations. Orchid density (D) reflects the number of orchids in the environment per true female, male capacity (c) is the number of times a male can mate before he becomes sperm depleted. Colours indicate the threshold orchid density that permits 0.01, 50% or 100% of the production level of the best-case scenario to be maintained, with best-case computed within each panel as daughters produced per female when $c = 30$ and $D = 0$. Persistence thresholds given by grey lines, above these populations are extinct. (B) Sex ratio predictions. Diploid populations do not adjust sex ratios as there is no production of males caused by matelessness, sex ratio is 1:1 everywhere below the persistence threshold (grey dash line), and the population is extinct above it. For haplodiploids, sex ratios are indicated by colour. Extinction (via all offspring becoming male) is given in grey. Note that no extinction (100% male) takes place.

$$f = 1; T_P = 0; s = 1.$$

Chapter 6: Museum records indicate male bias in pollinators of sexually deceptive orchids

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6.1 Introduction

For sexually reproducing populations, population persistence depends on the availability of males (and sperm; Holman and Kokko, 2013; Kokko, Heubel and Rankin, 2008; Rankin and Kokko, 2007). Females of some species are, however, able to reproduce entirely asexually. Hymenoptera (ants, bees, wasps, and sawflies) for instance, have a haplodiploid sex determination system. Typically, this means that haploid males are produced from unfertilized eggs, and females from fertilized eggs (via arrhenotokous parthenogenesis) (Normark, 2003). A sex determination system like this may offer a buffer when females are sperm deprived, or experience mating failure (Gaskett, Winnick and Herberstein, 2008). There

are also entirely female populations in Hymenopteran species where females have clonal daughters through thelytoky (Pearcy, Aron, Doums and Keller, 2004), though they are much rarer. Hence, sex determination systems like haplodiploidy may offer a buffer when females are sperm deprived or experience mating failure.

One scenario in which this buffer may be particularly important is when sexually deceptive orchids interfere with their pollinators' abilities to find and mate with female conspecifics, especially when they elicit sperm wastage (Chapter 3; Brunton Martin, Gaskett and O'Hanlon, 2020).

Sexually deceptive orchids mimic the sex pheromones of a female insect to attract a specific male pollinator (Schiestl, 2005); male pollinators mistake the flower for a female, and the deceptive plant is then pollinated during pseudo-copulation. Pseudo-copulation can be quite energetic, and in at least two orchid genera, true copulation and ejaculation occur. Work on *Lepanthes* orchids has found that fungus gnats vigorously copulate with flowers, and scanning electron microscopy suggested that spermatophores may be deposited (Blanco and Barboza, 2005). In this case, true copulation – rather than pseudo-copulation – may have occurred.

The Australian and New Zealand Tongue orchids, *Cryptostylis* spp., trick their wasp pollinator *Lissopimpla excelsa*, into pollinating them, such that

true mating events with ejaculation take place. Sperm presence was suspected by Edith Coleman in her ground-breaking work hypothesising the existence of sexual deception (Coleman, 1928), and has been confirmed by fluorescence microscopy (Brunton Martin et al., 2020; Gaskett et al., 2008). *L. excelsa* is a parasitoid wasp, a taxon in which males generally become totally or temporarily sperm depleted over several mating events (Boulton, Collins and Shuker, 2015; Damiens and Boivin, 2006; Sandanayaka, Chhagan, Page-Weir, Silva and Charles, 2011). Even if sperm production is infinite for this species, temporary sperm depletion after many mating events (with orchids or true females) may prevent a male from transferring seminal fluid containing sperm (Damiens and Boivin, 2006). Moreover, as most solitary wasp species are monandrous (Quicke, 2014; Ridley, 1993), a female who mates with a sperm depleted male may be sperm depleted for her entire lifetime.

Intriguingly, the vast majority of sexually deceptive orchids target haplodiploid pollinators (Chapter 1). Haplodiploid females that suffer from missed mating opportunities or sperm depletion can still reproduce – albeit all her offspring will be male. Indeed, populations with many females that receive insufficient sperm tend to produce unusually male-biased offspring sex ratios (King, 1987; Kranz, Schwarz, Giles and Crespi, 2000). I might

then expect that haplodiploid populations with high rates of sperm depleted females would have male biased sex ratios – which, in turn, would provide many males for both orchids and females. Are most extant sexually deceptive orchid pollinators haplodiploid because diploid pollinators lack resilience?

I constructed a model that confirmed my prediction that haplodiploid populations are better able to persist than diploid populations in the face of orchid sexual deception (Chapter 5). Strikingly, at orchid densities at which diploids went extinct or were unable to maintain ideal daughter production, haplodiploids were still able to persist and had a male-biased sex ratio (greater than 75% male). This means that orchid pollination and fitness need not be restricted by the costs they impose upon their pollinators, provided the pollinator is haplodiploid. Conversely, interference in haplodiploid pollinator reproduction may even create more pollinators for them.

Ideally, the prediction that sexual deception leads to male-biased pollinator populations would be tested with field surveys of male and female insects at sites with and without orchids. Unfortunately, in practice, these are extremely difficult as females of many solitary wasp species including *L. excelsa* are cryptic, poorly known, and can be difficult to catch or rear in

labs. Moreover, ichneumonid females typically have low parasitism rates (Frayssinet et al., 2019; Whitehouse et al., 2017) and thus obtaining an offspring sex ratio is very difficult, even if attempts are made to collect wild larvae that might be parasitised, or use larvae as ‘bait’ in traps. However, museum records and public sightings have been used in other studies to broadly estimate adult sex ratio of populations with some success (Bosque and Pacheco, 2019; Ford, Menzel, Menzel and Welch, 2002; Hornett, Charlat, Wedell, Jiggins and Hurst, 2009), though it is necessary to acknowledge and account for bias in samples. Here, I use museum records and public sightings to estimate the adult sex ratio of *L. excelsa* populations that do and do not live in sympatry with sexually deceptive *Cryptostylis* orchids. I hypothesize that exploited wasp populations that co-occur with orchids will have a male-biased adult sex ratio, whereas populations unaffected by orchids will have either a female-biased sex ratio (as is typical for parasitic hymenopterans (Godfray, 1990) or a 1:1 sex ratio (Hardy, 1994).

6.2 Methods

Record collection

In both Australia and New Zealand, *L. excelsa* is distributed broadly, while *Cryptostylis* orchids occur in patchy locations. I recorded the sex and location of *L. excelsa* specimens from entomological collections from museums (Te Papa, Auckland War Memorial Museum, Landcare, ANIC, The Australian Museum, QDPC Insect Collection and Queensland Museum) and digital records from social networks (“Atlas of Life in the Coastal Wilderness,” 2019.; “Atlas of Living Australia,” 2019; “BowerBird,” 2019; “Flickr,” 2019; “iNaturalist,” 2019; “NatureShare,” 2019; “Questagame,” 2019) (e.g. iNaturalist at inaturalist.org “iNaturalist,” 2019) and online Atlases (e.g. Atlas of Living Australia at ala.org.au “Atlas of Living Australia,” 2019) across Australia and New Zealand (n = 626). For comparison, I identified and recorded the sex and location of a sister ichneumonid species, *Echthromorpha intricatoria*, which lives in the same areas (with and without orchids) as *L. excelsa* and shares a host (typically *Helicoverpa* larvae; n = 191 (Berry and Walker, 2004), and similar general life history. To my knowledge, *E. intricatoria* has never been recorded as a pollinator of sexually deceptive orchids, despite considerable research effort to identify the pollinators of Australian orchids in similar habitat (Hopper

and Brown, 2007; Phillips et al., 2017; Reiter, Bohman, Flematti and Phillips, 2018; Reiter et al., 2017). I therefore expect that the presence of orchids would affect the adult sex ratio of *L. excelsa*, but not *E. inticatoria*. The presence of orchids was determined using records from the Atlas of Living Australia and Herbarium records (n = 4,044).

Reducing bias

Many of the insect record localities were not specific and coordinates were not provided. As such, I used approximate coordinates found using the written locations. Where locations were too broad (e.g. “Sydney” versus “Macquarie”), they were excluded from the analysis. To avoid collection bias, I excluded male wasps that were found on orchids, had orchid pollinia attached to their abdomen, or were collected by orchid researchers.

Similarly, I excluded females found in agricultural locations to avoid bias by insect collectors focussing on parasitoid hosts, e.g. those investigating biocontrol in cotton (Baker, Tann and Fitt, 2008; Lawrence, Whitehouse, Wilson and Fitt, 2005; M E A Whitehouse, Wilson and Fitt, 2005).

Where possible, I recorded the month and year of collection. Because very few years had multiple observations within them, they were lumped into decades. I carried out an initial generalised linear model to detect bias in sampling over time, i.e. whether sexes were more likely to be caught in a

decade or month. Because male ichneumonids have shorter lifespans than females (Coskun and Kayis, 2017), I would expect them to be able to be collected only during certain times of the year. Exploratory plots revealed that between June and October, there were no records of males for either *L. excelsa* or *E. intricatoria* (Figure 2). As male ichneumonids do not live as long as females, and may only be present during mating season (Quicke, 2014), I excluded observations outside of the mating season from subsequent cluster analyses. I report the number of observations and the proportion of males and females collected by decade and month (Figure 1 and Figure 2).

Determining groups

In order to determine whether records came from sites with or without orchids, I used the Geosphere package in R (Hijmans, 2019) to carry out hierarchical cluster analysis based on the latitude and longitude of each observation. Every observation was clustered into a group based on their distance from one another, such that all observations in a group were within a 15km radius. A group was defined as “with orchids” if it was within 15km of an orchid observation. Finally, the sex ratio was only calculated for groups with 4 or more observations, and for groups where the sex ratio was greater than 0 and less than 1.

Ultimately, my observations were reduced to 130 records of *E. intricatoria* (n (groups with) = 3; n (groups without) = 7) and 326 records of *L. excelsa* (n (groups with) = 10; n (groups without) = 23).

Testing for a difference in sex ratio

I calculated the sex ratio for each group of specimens and carried out a generalized linear model using a binomial distribution to determine whether there was a difference in sex ratio between species at sites with and without orchids, blocking by country. Sex ratio was weighted by the number of observations in each group. Multiple comparisons were accounted for using False Discovery Rate (FDR) correction.

6.3 Results

Overview of specimens

Almost all records (85%) were obtained in spring-summer (southern hemisphere, November to May; Figure 2). Outside of these months, only females were recorded (15% of records) for both *L. excelsa* and *E. intricatoria*. Notably, records of flowering *Cryptostylis* orchids (n = 109) fell between October and March. Of the records with information on collection year, most were collected in 2010 (44%). 1970 accounted for 15% of records, and 1980 for 18%. The sex of the specimen did not vary

significantly with year (Figure 1A). I note that there is a large peak of observations in the last decade, with the advent of digital records (Figure 1B).

Differences in sex ratio

I found that wasp species and orchid presence had a significant effect on sex ratio (averaged over country, Figure 3 A). *L. excelsa* at sites with orchids were significantly more male-biased than *excelsa* at sites without orchids (FDR corrected p value = 0.001). *L. excelsa* was also more male-biased than *E. intricatoria* at sites both with and without orchids (FDR corrected p values: 0.02 and <0.01 , respectively). Sex ratios from all other groups were not significantly different. There was no significant interaction between species and country or orchid presence and country, but across all species, sex ratios in New Zealand were higher than in Australia (Figure 3 B).

6.4 Discussion

My previous model about sexually deceptive orchids that elicit sperm wastage predicted that haplodiploid pollinators were better able to withstand the effects of deceptive orchids than their diploid counterparts. It suggested that when orchids “steal” sperm, this drives females to

produce more sons, and thus a male-biased sex ratio. This then could provide an abundance of mates for females and pollinators for orchids – thereby buffering the effects of extreme sexual deceit. Here, I support this by reporting that for museum and digital records of an exploited pollinator (*L. excelsa*) that lives in sympatry with orchids, there is a significantly more male biased sex ratio (70% male) than for the same pollinator at sites without orchids (40% male); and a sister ichneumonid at sites both with (40% male) and without (30% male) natural populations of orchids. I note that these results heavily rely on the accuracy and breadth of the records available to us. Though I attempted to reduce the effect of bias as much as possible, it may well be the case that male bias at sites with orchids is a consequence of the ease of finding males around areas where orchids occur. I have attempted to test this in the field but could not yield enough data to determine a sex ratio. Over six weeks in peak season, I trapped with sugar spray (20%) baited (Jacob and Evans, 1998; Rogers and Potter, 2004) and un-baited yellow sticky traps at 3 sites with and 3 sites without natural populations of orchids; but I caught only 4 male and 1 female *L. excelsa*. Future work might investigate the effect of unmatedness on exploited populations in a laboratory setting, or even in controlled field environments. It may also be possible to calculate offspring

sex ratio using larval hosts of *L. excelsa* as bait, although this species has particularly low rates of parasitism (Baker et al., 2008; Berndt, Berry, and Brockerhoff, 2006; Frayssinet et al., 2019; Johns and Whitehouse, 2004; Whitehouse et al., 2017).

If the results of this museum and digital record survey are representative of the natural populations, orchid presence may indeed influence the sex ratio of pollinators. Sex ratios of haplodiploid populations may be altered due to the amount of mated and unmated females in a population.

Rautiala et al.(2017) constructed a model that predicted that once the number of females in a population reached high enough levels of unmatedness, and any females in the population that were mated were selected to produce only daughters, populations became male-biased. This is because under haplodiploidy, unmated females can reproduce, albeit they are constrained to produce only sons. Notably, at sites with orchids, this survey found that records of the pollinator were 70% male. My own model on this system found that *Cryptostylis* orchids drive levels of female unmatedness that shift sex ratios to between 75% and 95% male: low mate availability in one generation leads to a higher proportion of spermless females that same season – either due to males becoming sperm depleted during encounters with orchids, or simply encountering orchids instead of

females. Even if the proportion of unmated females is small, the presence of a fraction of females that are constrained to produce males will affect population sex ratio – allowing for an increase in males, and thus an increase in mating opportunities for the next generation. With higher orchid densities, the sex ratio should be more male biased for the population to persist. At sites with orchids, *L. excelsa* was significantly more male biased than a sister ichneumonid, *E. intricatoria* that occurred at the same sites. This indicates that orchid presence may be an important factor in predicting sex ratio in exploited populations.

My previous model suggests deception has more significant impact when the population is protandrous (Chapter 5), as is thought to be the case in ichneumonid wasps such as *L. excelsa* (Quicke, 2014). This is because orchids have a chance to capture males' attention, and sperm, before virgin females emerge. Consistent with this, I found there are more records of males (*E. intricatoria* and *L. excelsa*) in November, at the start of the season, and *Cryptostylis* orchids have already begun flowering at this point (Figure 2). This suggests the records I used provide at least a general indication of natural population dynamics.

Both *E. intricatoria* (at sites with and without orchids) and *L. excelsa* (at sites without orchids) had a slightly female-biased adult sex ratio.

Hymenopteran parasitoids are typically predicted to have an even adult sex ratio (Fisher, 1999), but can become heavily female-biased (Godfray, 1990, 1994; Hamilton, 1967). Understanding variation in sex ratio is important because it can impact the fitness of individuals, and is dependent on their breeding and sex-determination systems (Pipoly et al., 2015). Adult sex ratios may become biased due to mortality differences in juveniles or adults between males and females; or difference in male and female migration and dispersal (Székely, Liker, Freckleton, Fichtel, and Kappeler, 2014). Bias might also be a consequence of reproductive parasites such as *Wolbachia* in many hymenopterans and other insects (Gerth, Geibler and Bleidorn, 2011; Klopstein, van Der Schyff, Tierney and Austin, 2018; Zchori-Fein, Gottlieb and Coll, 2000), as they kill males (O'Neill, Hoffmann and Werren, 1997; Werren, 1997) and, in some species, initiate thelytokous parthenogenesis (Farrokhi, Ashouri, Shirazi, Allahvari and Huigens, 2010) – skewing the sex ratio toward females.

These findings demonstrate that the impacts of orchids on their pollinators may well be underestimated. These preliminary patterns support the theory that sexually deceptive orchids can manipulate the sex ratio of their haplodiploid pollinators. Further research on individual responses to sperm limitation broadly and in the context of sexual deception are needed to

understand this mechanism better and empirically confirm any changes in pollinator sex ratio. I also highlight the utility of museum and digital records for this type of work: as people now have excellent cameras in their pocket, observations that are reasonably simple to classify are becoming increasingly available. This is a valuable tool that, combined with historical records, could help us quantify biodiversity in the future.

6.5 Tables and figures

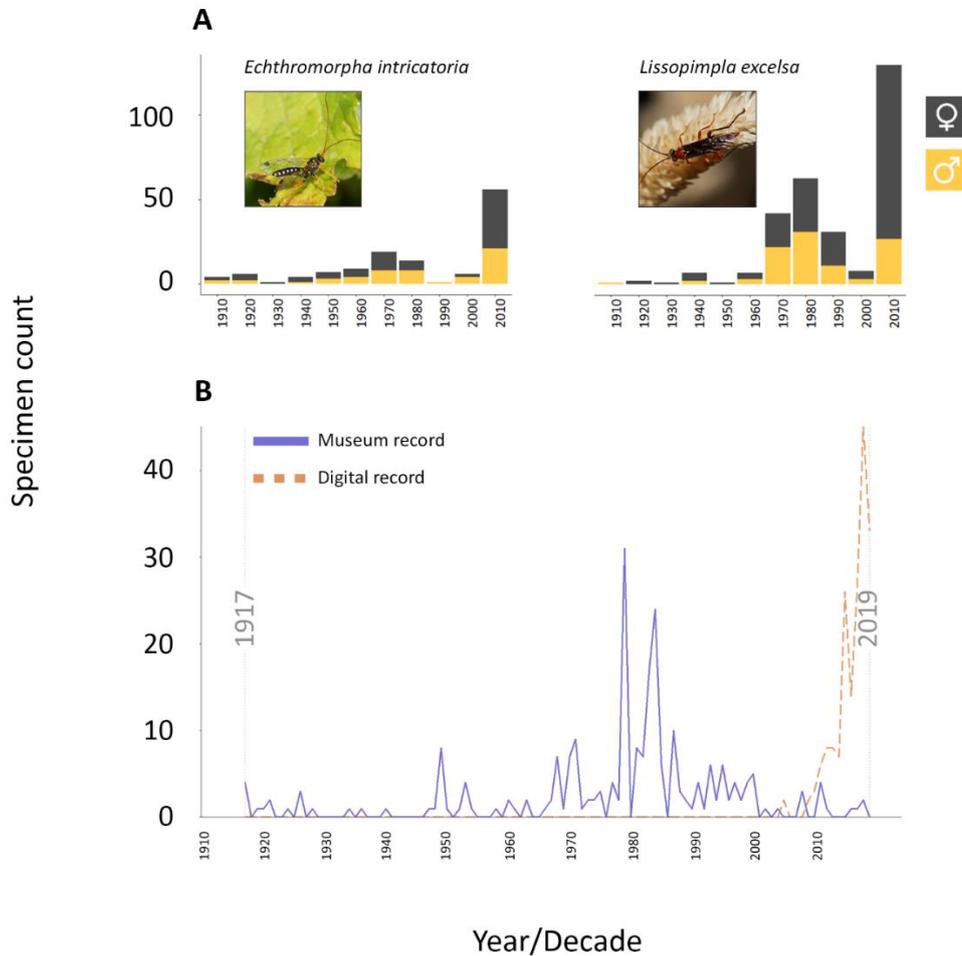


Figure 1: (A) The number of records of male and female specimens by decade separated by species, the non-orchid pollinator, *Echthromorpha intricatoria* (left) and *Cryptostylis* pollinator, *Lissopimpla excelsa* (right). Species images exemplify digital records: sourced from iNaturalist. Images taken by Reiner Richter and reproduced here with permission. (B) All record counts by each year, with dotted line indicating the range of years for all records used for this survey (between 1917 and 2019).

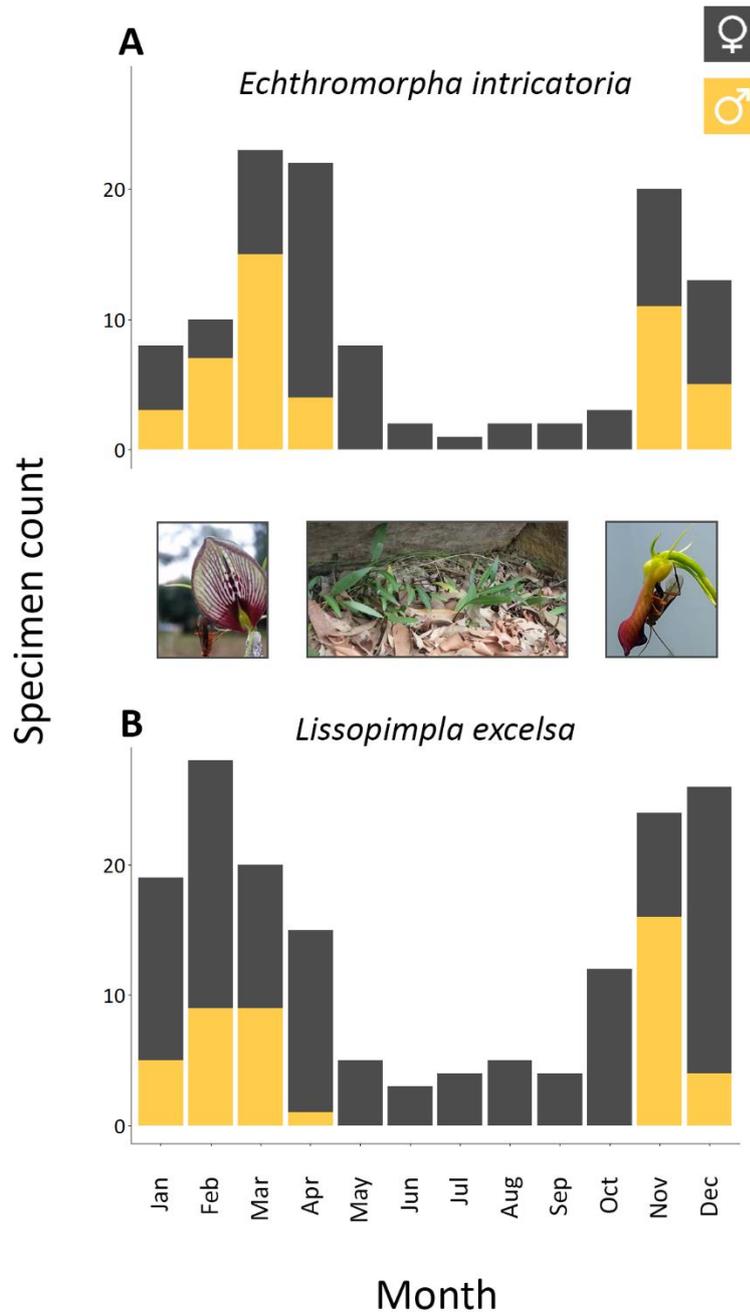


Figure 2: The number of records of male and female specimens of (A) the non-pollinator, *Echthromorpha intricatoria* and (B) the *Cryptostylis* pollinator, *Lissopimpla excelsa* (pictured). The flowering period of *Cryptostylis* is highlighted by the centre images, records of *Cryptostylis* indicate they are in flower between Jan-Feb and Oct-Dec.

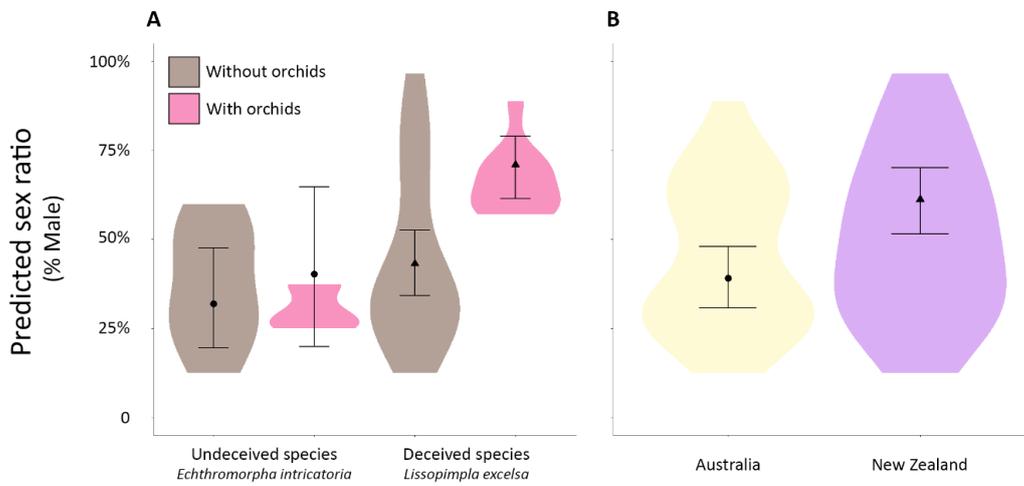


Figure 3: (A) Predicted sex ratios from generalised model for two species of ichneumonid parasitoid wasps from populations with (pink) or without (brown) sexually deceptive orchids. These wasps are either deceived by sexually deceptive orchids (*Lissopimpla excelsa*, triangle points) or not (*Echthromorpha intricatoria*, circle points) but share a larval host and similar life history. (B) Predicted sex ratios across all species at Australia (yellow, circle point) and New Zealand (purple, triangle point). Bean behind points are the distribution curves of the sex ratios from raw data, error bars indicate a 95% confidence interval.

Chapter 7:

Better pollinators are resilient - haplodiploid dupes improve orchid fitness.

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Sciences*

7.1 Introduction

There are a variety of ways in which a plant may be deceptive. When deception is involved with pollination, deceptive strategies are tightly linked to plant fitness (de Jager and Peakall, 2016, 2019; Walsh and Michaels, 2017). One common strategy of deceit is generalised food deception (Thakar, Kunte, Chauhan, Watve, and Watve, 2003), in which plants take advantage of an animal's instinctive foraging behaviour. For instance, the Crimean Iris, *Iris lutescens*, deceives pollinators through the bright colours of its petals: falsely indicating the presence of nectar (Imbert et al., 2014). Similarly, the green-winged orchids, *Anacamptis morio* and

A. pyramidalis, trick their pollinator into visiting it through floral scent and colouration (Fantinato, Vecchio, Baltieri, Fabris and Buffa, 2017). These food deceptive species often depend on naïve pollinators, as otherwise, they might learn to avoid deceptive flowers (Smithson and Gigord, 2003). Other deceptive strategies include brood-site or shelter imitation. Brood-site deceptive flowers, such as the nectarless, fly-pollinated *Pleurothallis johannensis* and *P. fabriobarrosii* (Borba and Semir, 2001), mimic the oviposition sites of animals, attracting female insects to lay their eggs.

Arguably, the most intriguing form of deceit is, however, that of sexual deception. In sexual deception, the exploitative plant will harness their pollinator's mating behaviour to their advantage: imitating the mating signals of their pollinators. In this way, the male pollinators mistake the flower for a female and pollinate the deceptive plant, typically through 'pseudo-copulation' (Schiestl, 2005). Pollination by sexual deception is most commonly found in orchids (approximately 400 species of orchids are sexually deceptive (Gaskett, 2011; Schiestl et al., 2000), but it is also recorded in one species of daisy (Asteraceae; *Gorteria diffusa* ; Ellis and Johnson, 2010), and an iris (Iridaceae; *Iris paradoxa* ; Vereecken et al., 2012). Deceptive orchids typically obtain half the pollination rate than

that of their rewarding counterparts (Tremblay, Ackerman, Zimmerman and Calvo, 2005). For instance, studies comparing rewarding and deceptive orchids in Africa and Europe found that the pollination rates of rewarding orchids were approximately double the rate of that of deceptive orchids (Europe: 63.1% in rewarding vs 27.7% in deceptive; Neiland and Wilcock, 1998; Africa: 64.8% vs. 25.2% ; Johnson and Bond, 1997). Sexual deception is associated with even lower pollination rates (average $\sim 30\%$ over orchids from Europe, and Temperate/Tropical southern hemisphere; Neiland and Wilcock, 1998). *Cryptostylis* orchids, however, have exceptionally high pollination rates of up to 90% (mean $\sim 70\%$; Gaskett, 2011; Schiestl, Peakall and Mant, 2004), with exciting implications for the long-term sustainability of this pollination strategy, given the potential costs of deception to pollinators.

Although most sexual deception is assumed to elicit pseudo- rather than true copulation, actual copulation (with ejaculation) may occur in *Lepanthes* orchids (Blanco and Barboza, 2005), and is demonstrated in *Cryptostylis* orchids (Figure 1; Brunton Martin, Gaskett and O'Hanlon, 2020; Coleman, 1928; Gaskett, Winnick and Herberstein, 2008). The five *Cryptostylis* orchid species in Australia and Aotearoa/New Zealand attract a shared, single pollinator (males of the orchid dupe wasp, *Lissopimpla*

excelsa) through sexual deception – mimicking the sex pheromones and appearance of females (Bohman, Weinstein, Phillips, Peakall, and Flematti, 2019; Gaskett, 2012; Gaskett and Herberstein, 2009). Through this process, they trick males into mating with them and ejaculating their sperm – while also collecting the pollinia from the orchid. Sperm loss is not trivial for populations of *L. excelsa*: males of solitary parasitoids like this species may become sperm depleted after a few mating events (even if only temporarily; Brunton Martin et al., 2020).

My previous model (Chapter 5) proposed that sexually deceived pollinators, such as *L. excelsa*, are better able to cope with the costs of exploitation because they are almost always haplodiploid (Chapter 1). Haplodiploidy permits females to reproduce with or without sperm, although when she reproduces without sperm, all her offspring will be male. I found that it may be advantageous for orchids to elicit pollinator ejaculation as preventing a large proportion of females from obtaining sperm creates a male-biased sex ratio in the population. This bias provides more males to act as pollinators. Haplodiploidy also helps pollinator populations persist when there are high costs. However, this model addressed the consequences for pollinator populations and did not address the problem from the orchid's perspective. If orchids create more males in

an exploited population, I might expect that orchids that elicit ejaculation from haplodiploids would experience improved pollination success. Thus, there may be strong selection for haplodiploid pollinators, driven by fitness benefits for both the orchid and the pollinator. I propose that *Cryptostylis* orchids achieve this by eliciting sperm wastage from haplodiploid pollinators.

Here, I consider the effect of haplodiploidy and sperm wastage on orchid pollination success. Building on my original model, I contrast the pollination success of two theoretical populations of orchids: one targeting diploids and one targeting haplodiploids. I predict that orchids that target haplodiploid pollinators will have higher pollination rates than orchids with diploid pollinators.

7.2 The model

This model is an extension of my previous model and based on the life-history traits and mating behaviours of solitary parasitoid wasps (Abe, 2019; Avila, Withers and Holwell, 2017; Quicke, 2014): females are monandrous, and males polyandrous. Males will continue to mate once sperm depleted, and typically emerge before females (protandrous).

However, I vary protandry here to explore how it might alter pollination

rates. Full summaries of mating and reproduction of pollinators in the original model is presented in Figure 2 and Figure 3, respectively.

Here, I expand on my original model by tracking pollen on males and the gain and loss of pollen on orchid flowers. As per the previous model, I assume that there are D orchids within a unit area of habitat. I track pollination success over T_{\max} generations (I set T_{\max} to 50 so that the population always stabilises or goes extinct) and each generation occurs over a season of length S . As per the Euler method, days in each season are divided into 100 steps in order to have each time step short enough to represent a discretized approximation of differential equations. Each step is assumed to yield maximally one encounter for each focal individual.

Tracking males as pollen vectors

At the beginning of each season, males of the pollinator species emerge. Males have a mating capacity of c matings before becoming sperm depleted, although they will continue to mate once they have mated maximally (see Female reproduction: “Does male have sperm?” in Figure 2). In each time step, males can encounter with orchids or females (if they have emerged, this depends on the set level of protandry. See Male reproduction: “Have females emerged yet?” in Figure 2). Males may have several mating events in one day: this depends on their mate search rate,

s , which I vary. If they can, males will spend sperm with each encounter, pushing them one step closer to sperm depletion.

Additionally, at each time step ($t = 1$ onwards), males gain pollen from orchids when they encounter virgin ('00') flowers. Males cannot gain pollen if they already have pollen. The number of encounters between males with no pollen and virgin orchid flowers is

$$G_i(t) = s A_i(t) O_{00}(t) dt$$

where I use the letter G to denote pollen-gaining encounters; A to denote pollen-absent males; and O to denote orchid flowers.

Males will also lose pollen when they encounter flowers that have been visited but not gained pollen ('01') or virgin flowers ('00'), the numbers calculated as

$$V_i(t) = s P_i(t) O_{00}(t) dt$$

$$R_i(t) = s P_i(t) O_{10}(t) dt$$

Where V denotes the number of 'virgin' orchids becoming pollinated; R the number of orchids that have had pollen removed becoming pollinated; P to denote males with pollen; and O to denote orchid flowers.

I can hence calculate the total number of males that had pollen but have now lost it as

$$L_i(t) = s P_i(t) O_{00}(t) + s P_i(t) O_{10}(t) dt$$

where the letter L_i is used to denote pollen-losing encounters.

The temporal dynamics is then computed, for males with pollen as

$$P_i(t+1) = P_i(t) - L_i(t) + G_i(t)$$

And for males without pollen as

$$A_i(t+1) = A_i(t) + L_i(t) - G_i(t)$$

Tracking pollination status

All members of *Cryptostylis* can produce a single flower raceme up to 1 metre tall and bearing up to 30 flowers, which open sequentially as older flowers become pollinated or senesce (Graham, 1976). As in the wild, in this model, males can only gain pollen from a previously unvisited orchid flower ('virgin'). For simplicity, orchids once pollinated cannot be pollinated again. The number of orchids is initialised with D orchids at $t = 1$. I track three categories of orchids: virgin orchids ('00'); orchids with pollen removed ('10'); and fully pollinated orchids with both pollen deposited and removed ('11'). Encounters of 00 or 10 orchids with a male with pollen will bring them to a pollinated state.

Orchids lose pollen when a male without pollen visits them; this number is the same as $G_i(t)$. Orchids of state 00 or 10 will become pollinated when they encounter males who have previously encountered a virgin orchid (00). I calculate this as

$$O_{00}(t+1) = O_{00}(t) - Gi(t) - Vi(t)$$

$$O_{10}(t+1) = O_{10}(t) + Gi(t) - Ei(t)$$

$$O_{11}(t+1) = O_{11}(t) + Ei(t) + Vi(t)$$

At the end of the season, the proportion of pollinated orchids (pollination rate) is

$$\frac{\sum_t O_{11}(t)}{\sum_t (O_{00}(t) + O_{10}(t))}$$

Here, I present the pollination rate of orchids for a stable (or extinct) population of pollinators.

7.3 Results and discussion

My previous model found that for both haplodiploids and diploids, as male capacity increases, populations can tolerate higher orchid densities. The model also found that where diploid populations go extinct, haplodiploid populations persist. Even where both diploid and haplodiploid populations persist, offspring production by diploid populations is lower than that of haplodiploids. Here, I show that orchid populations that target haplodiploid pollinators ('haplodiploid-pollinated orchids') consistently achieve higher pollination rates than if they were to target diploid pollinators ('diploid-pollinated orchids'; Figure 4). Orchids that target haplodiploids still maintain higher pollination rates under realistic situations for *Cryptostylis* orchids, where females of the pollinator species lay an egg each day, males have several mating events in a day, and females emerge later than males. The highest pollination rate achieved under these assumptions for diploids is 59% versus 71% for haplodiploids (improved almost 1.3×; Table 1).

The improved orchid pollination rates are associated with changes in the pollinator sex ratio. At higher orchid densities, diploid-pollinated orchids have lower pollination rates than haplodiploid-pollinated orchids. Orchid populations also persist for longer when pollinated by haplodiploids (above

50% male line; Figure 4). Pollination success also appears to be impacted, somewhat, by the efficiency of male mate searching. Higher male mate search rates result in higher pollination rates in haplodiploid-pollinated orchids (Figure 5 A compared with B, and see max. and min. values in Table 1, comparisons 2 and 3). These higher male mate search rates also decrease persistence for both haplodiploid and diploid pollinators.

As per my first model, haplodiploid pollinators are, however, better able to persist when their search rate is higher. Increasing protandry only marginally impacts pollination rates, with longer protandry resulting in increased pollination rates for both haplodiploid-pollinated and diploid-pollinated orchids. However, the effect is more substantial in haplodiploid-pollinated orchids (Figure 5 E compared with G, and see max. and min. values in Table 1 comparisons 2 and 4). Both can be explained intuitively: with longer protandry, there is more time when searching males only encounter orchids. With high mate search efficiency, males can find many orchids to mate within one day – increasing the chances of an orchid becoming pollinated.

These results, paired with my previous model, suggest that it is advantageous for orchids that target haplodiploids to elicit ejaculation. The male-biased sex ratio that arises in response to sexual deception in

haplodiploid populations creates more pollinators for orchids and thus improved pollination rates. This fitness advantage is absent when the pollinator is diploid. The pollination rates I report here are in line with those found by other researchers of sexually deceptive *Cryptostylis* orchids (50-90%; Gaskett and Herberstein, 2006; Schiestl et al., 2004).

In contrast, pollination rates are comparatively lower for the diploid-pollinated sexually deceptive *Lepanthes* orchid that also likely elicits ejaculation in their fly pollinator, at between 12 and 40% (Blanco and Barboza, 2005; Calvo, 1990). These pollination rates are also lower than I estimate here. Hence, it is prudent to note that both models assume the pollinator is an ichneumonid wasp, and the only difference between pollinator populations is diploidy and haplodiploidy. Future models might better compare diploid and haplodiploid populations using the traits that are unique to diploid pollinators (e.g. *Lepanthes* orchids have fungus gnat pollinators, where males and females live for approximately ten days, but females have high fecundity (Gillespie, 2019). Intriguingly, fungus gnats have temperature-dependent sex ratios. In colder climates, where sexually deceptive *Lepanthes* orchids grow, fungus gnat offspring are male-biased (Nigro, Campos, and Perondini, 2007). This form of sex allocation may allow this diploid population to persist.

This model suggests that by targeting, and eliciting sperm wastage, from haplodiploid pollinators, sexually deceptive orchids can enjoy improved pollination success, while limiting the risk of extinction, for both themselves and their pollinator. The manipulation of a resilient, haplodiploid pollinator appears to contribute, somewhat, to the extraordinary success of *Cryptostylis* orchids.

By re-examining the mating systems, behaviour and sex ratios of known diploid and haplodiploid pollinators, future research may clarify if this mechanism is broadly responsible for the long-term evolutionary maintenance of deceptive relationships. Generally, taking a close look at the traits of exploited species might determine whether resilience allows for persistence and improved exploiter fitness in other asymmetric, co-evolutionary relationships.

7.4 Tables and figures

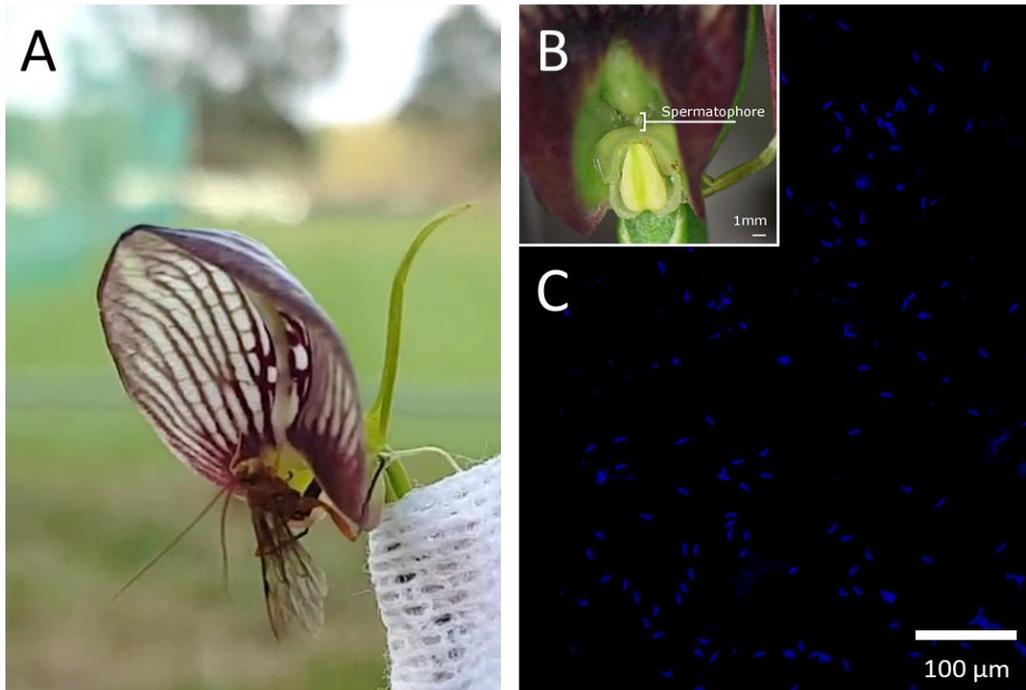


Figure 1: To ensure pollination, *Cryptostylis erecta* tricks its pollinator, *Lissopimpla excelsa* (A) into mating with it and inadvertently transferring the pollinia, whilst also depositing a spermatophore (B) that contains tens of thousands of sperm (C).

and text: Variables that change in the model — — — — — Return for the next day/iteration ($t + 1$) in the season

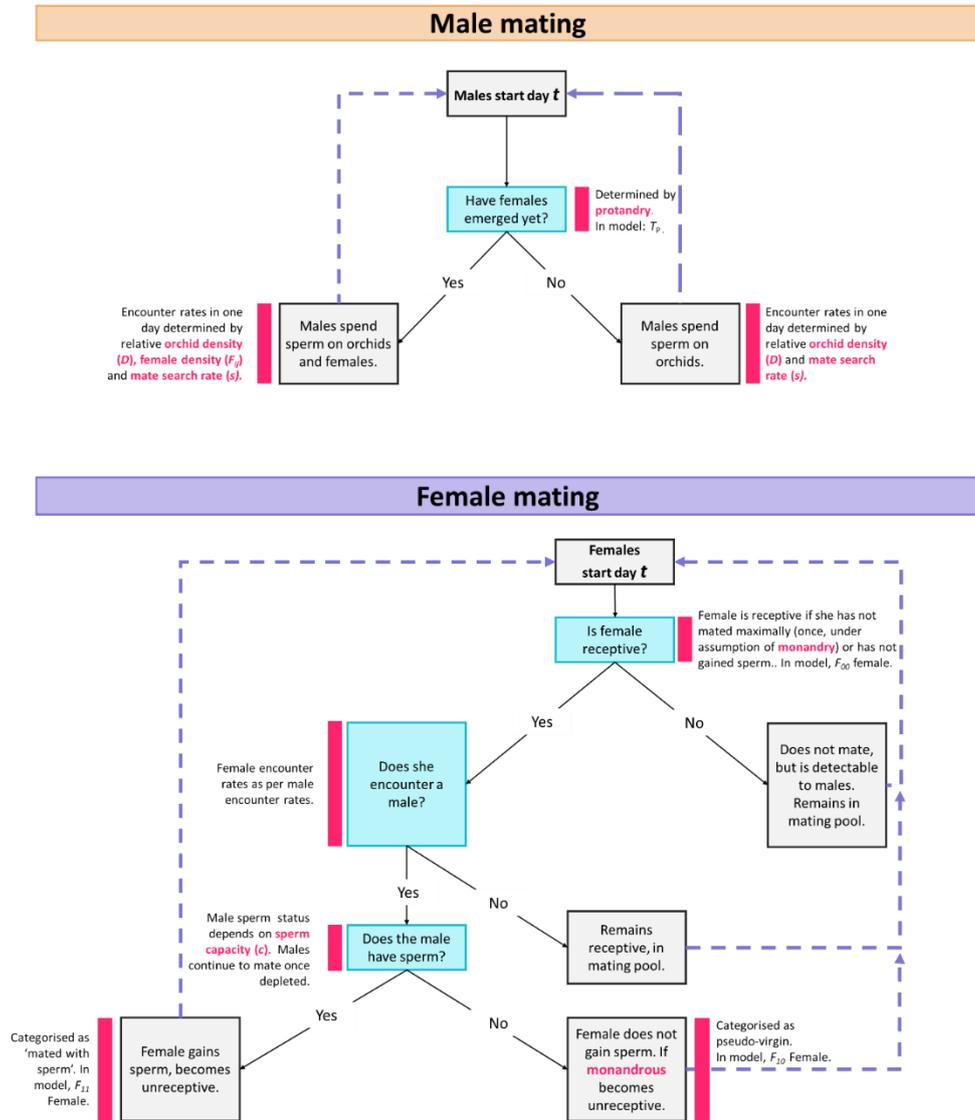


Figure 2: How male and female mating in a season (season length set at the beginning of model iteration) works in original model (Chapter 5). Pink boxes and writing indicate variables that change in the model, either set at the beginning of the model run for all generations (e.g. the sex-determination system of the population or protandry) or varies between generations because of a previous iterations outcome (e.g. female density, determined by the number of daughters from one generation to the next).

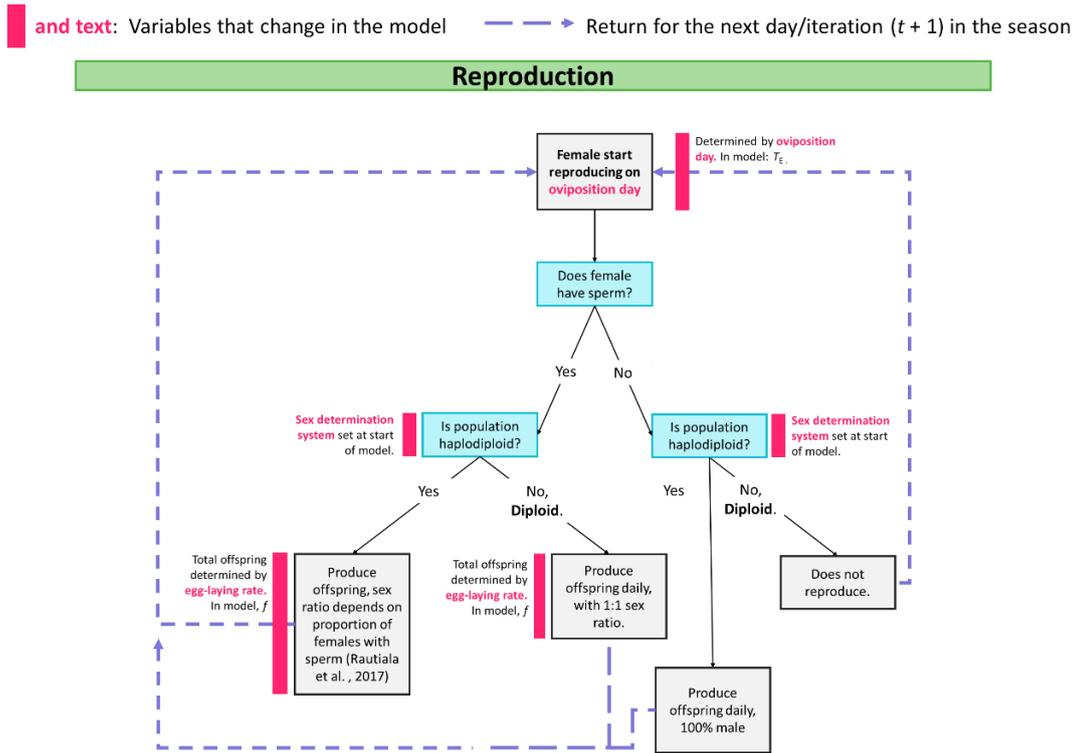


Figure 3 How reproduction in a season (season length set at the beginning of model iteration) works in original model (Chapter 5). Pink boxes and writing indicate variables that change in the model, either set at the beginning of the model iteration (e.g. oviposition day). Note that the reproduction of haplodiploid females with sperm varies depending on female unmatedness as per the analysis of Rautiala, Helanterä and Puurtinen, 2017.

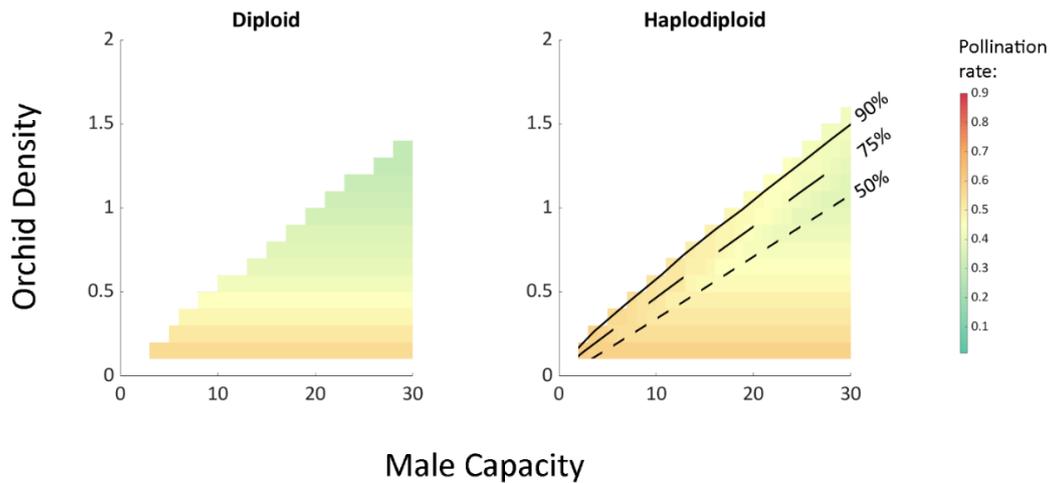


Figure 4: Pollination rates of orchids that target haplodiploid and diploid pollinator populations. Orchid density (D) reflects the number of orchids in the environment per true female, male capacity (c) is the number of times a male can mate before he becomes sperm depleted. The curves on the haplodiploid figure indicate the sex ratio of the pollinator population. Sex ratios are 1:1 short-dashed curve, below 75% underneath the long-dashed curve and below 90% underneath the solid curve. As diploid pollinator populations do not adjust sex ratios, and there is no production of males caused by matelessness, for these pollinators sex ratios are always 1:1. Pollination rate indicated with the colour scale. Note hotter colours (yellow and orange) throughout haplodiploid pollinated orchid indicate higher pollination rates.

$$f = 1; T_P = 5; s = 5.$$

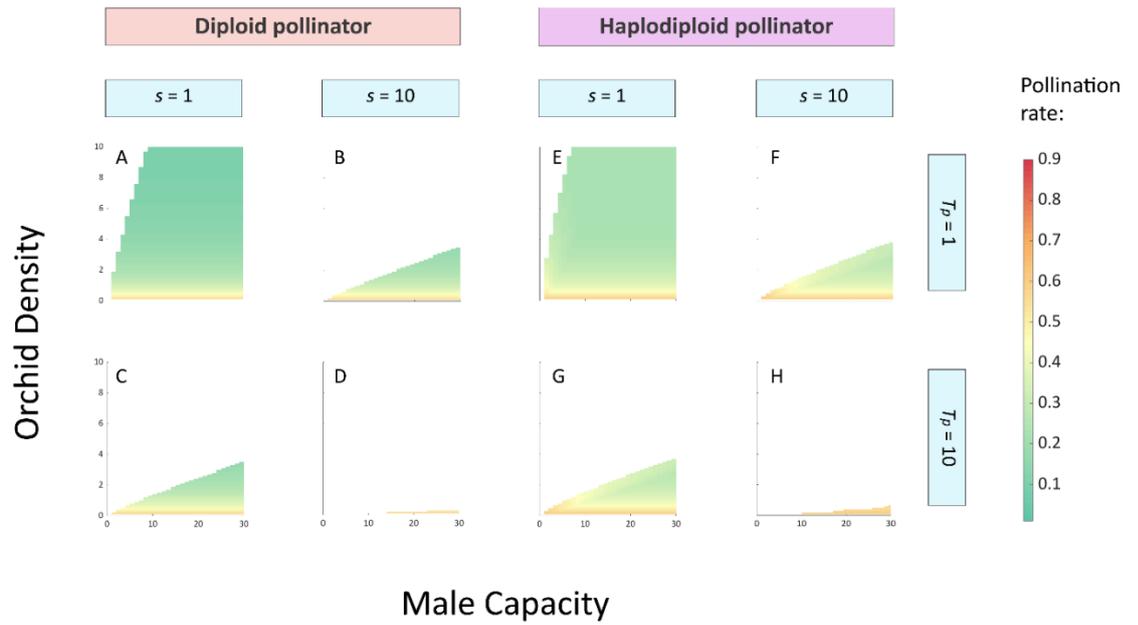


Figure 5: Pollination rates of diploid (left; A - D) and haplodiploid (right; E - H) populations for decreasing levels of protandry (T_p) and mate search efficiency (s) while fecundity (egg production, f) remains constant at 1. Orchid density (D) reflects the number of orchids in the environment per true females, male capacity (c) is the number of times a male can mate before he becomes sperm depleted. Pollination rate indicated with colour scale.

A/E: $s = 10$, $T_P = 10$; B/F: $s = 10$, $T_P = 1$; C/G: $s = 1$, $T_P = 10$; D/H: $s = 1$, $T_P = 1$

Table 1: The maximum and minimum pollination rates achieved for orchids with a diploid pollinator versus those with a haplodiploid pollinator. Best-case scenario (for the pollinator) from which I can derive a pollination rate (i.e. orchid density is > 0) is a density of 0.1 orchids for every one female and high male capacity ($c = 30$). Worst-case scenario is the highest orchid density and lowest male capacity that does not lead to pollinator extinction (and as such, a pollination rate would not be able to be determined).

Comparison	Figure reference	Diploid pollination rate		Haplodiploid pollination rate	
		<i>Best-scenario</i>	<i>Worst-scenario</i>	<i>Best-scenario</i>	<i>Worst-scenario</i>
1	Figure 4	59%	33%	71%	49%
2	Figure 5 A vs E	58%	18%	59%	23%
3	Figure 5 B vs F	58%	24%	65%	40%
4	Figure 5 C vs G	58%	22%	61%	31%
5	Figure 5 D vs H	45%	52%	62%	57%

Chapter 8: General discussion

8.1 Synthesis

How do exploited species persist? It was clear from the outset that a complete explanation for this conundrum was not possible to achieve in just three years. This thesis, however, exploits two charismatic, native species to make great strides towards an explanation. *Cryptostylis* is an extreme deceiver, and we can easily observe that its pollinator, *L. excelsa*, mates quickly and enthusiastically with orchid flowers (Figure 1). These characteristics make it an excellent first model species for testing the idea of *resilience* as a mechanism to help explain the persistence of costly relationships and better understand this fascinating topic.

Previously, research seeking to answer this question focused on: counteradaptations, whereby exploitation drives selection for both the exploiter and the exploited party; contexts where indirect fitness benefits compensate for the costs of exploitation; or situations where the chances of the exploitative interactions are low. In many cases, however, these explanations are not adequate – particularly in terms of the success of

sexually deceptive orchids (reviewed in Chapter 1). Orchid species that provoke the most extreme pollination behaviour, like *Cryptostylis*, have the



Figure 1: Before using an insect rearing tent for field experiments, upwards of 10 wasps would visit an exposed *Cryptostylis* flower at one time. Video still of presented *Cryptostylis subulata* flower from the first day of fieldwork, January 5th 2017, at Christie Park, Sydney.

highest likelihood of fooling insects into landing on flowers (Gaskett, 2011), and obtain extraordinarily high pollination rates and fitness (Gaskett and Herberstein, 2006; Gaskett, Winnick and Herberstein, 2008; Schiestl, 2005). They also, however, impose the highest costs on pollinators (ejaculation and sperm wastage). Even if there are apparent fitness benefits, selection for such extreme deception should weaken if it imposes unsustainably high reproductive costs on pollinators (Brockhurst and

Koskella, 2013; Hembry, Yoder and Goodman, 2014). This thesis presents the idea that the insects that seem to be most vulnerable to orchid interference are also those that can cope with deception through pre-existing life-history traits. These traits confer *resilience* to costs imposed by exploiters and allow populations to persist in the face of extreme deceit. This thesis explores a putative resilience trait, haplodiploidy, and how orchid-imposed costs might interact with haplodiploidy to change population dynamics and shape individual behaviour and morphology. I quantified the costs for *L. excelsa* males that mated with *Cryptostylis* orchids (Chapter 4) and explored the consequences for the pollinators at both an individual and population level (summarised in Chapter 2). Mathematical models were used to test whether an inherent trait, haplodiploidy, permitted *resilience* against the costs of deception (Chapter 5), and I attempted to determine whether we could observe the outcomes of the model in nature (Chapter 6). Intriguingly, haplodiploidy does indeed allow populations to better persist in the face of extreme exploitation. Deceived haplodiploid populations that become sperm limited have a male-biased sex ratio and improved offspring production. Not only does this improve population persistence, but it even makes for a better pollinator (improving orchid pollination rates, Chapter 7). Additionally, I explored

whether the pollinators responded to the costs of deception, and whether those responses might be a response to the direct (counter-adaptations) or indirect (enhanced scramble competition arising from a male-biased population) impacts of orchids (Chapter 3).

8.2 Costs to a pollinator

The costs of sexual deception are typically considered negligible, due to the apparent low cost to fooled males (Schiestl, 2005). For many animals, however, sperm can be a limiting resource (Gaskett et al., 2008; Kokko, Heubel and Rankin, 2008; Lehtonen and Schwanz, 2018; Parker and Pizzari, 2010). The research presented in this thesis is one of two studies exploring sperm use in ichneumonid species, and the first to quantify the exact costs of sperm associated with sexual deception (Chapter 4, now in press in *Functional Ecology*). I found that the sperm stock of *L. excelsa* males with one known mating event with a sexually deceptive orchid was significantly less than males with fewer matings, suggesting that males may at least become temporarily sperm depleted during the mating season. Indeed, approximately a tenth of the males I captured were sperm depleted. Furthermore, I demonstrated that male *L. excelsa* vary their ejaculate size with their current sperm stock, as well as their previous

experience with orchids. This suggests that males can strategically allocate their sperm.

Differences in allocation might arise for three reasons. First, males might perceive orchids as low-quality females and therefore transfer less sperm (Reinhold, Kurtz and Engqvist, 2002). Second, males may change sperm allocation to optimise the total number of mating events they can obtain (Abe and Kamimura, 2015; Allen, Barry, Holwell and Herberstein, 2011; Parker and Pizzari, 2010; Wedell and Cook, 1999). Finally, the smaller ejaculates we observe may arise via pollinator learning. Pollinator learning in response to deception is not uncommon and is apparent in *L. excelsa* (Gaskett et al., 2008; Weinstein, Davis, Menz, Dixon and Phillips, 2016).

Costs of deception are not necessarily limited to sperm wastage. Sexually deceptive orchids may also inflict missed mating opportunities on males by wasting their search time (reviewed in Chapter 1). For example, male wasp pollinators of *Chiloglottis* orchids find females less attractive when females are among orchids. Mating events are crucial for female *N.*

cryptoides: they rely on males to provide them with food, transport to oviposition sites, and sperm. If a male chooses an orchid over a female, he reduces female fitness, which could impact population persistence. *L. excelsa* males will spend between 15 seconds and 15 minutes interacting

with an orchid. This is valuable time, assuming that ichneumonid males have a short lifespan (approximately 30 days (Ebrahimi, Sahragard, Talaei-Hassanloui, Kavousi and Chi, 2013; Korenko, Potopová, Satrapová and Pekár, 2016) and a small window of activity (6 am to 12 pm with which to search a maximum range of 600m (Weinstein et al., 2016) that may contain two types of potential mates: orchids or females.

8.3 Population impacts

In the last decade, it has become increasingly apparent that sperm and male availability is an important driver of population dynamics (Dyson and Hurst, 2004; Galliard, Fitze, Ferrière and Clobert, 2005; Kamimura, 2012; Kokko and Rankin, 2006; Lehtonen and Schwanz, 2018; Parker and Pizzari, 2010; Réale, Boussès and Chapuis, 1996; Reinhold et al., 2002; Rice, 2000; Wedell, Gage and Parker, 2002). In the case of sexual deception, deceived males may well become sperm depleted (Chapter 4), and I hypothesised that haplodiploidy might buffer the effects of sperm limitation (Chapter 1). Under this assumption, I constructed a mathematical model to test, over evolutionary time, whether deceived haplodiploid populations were better able to persist in the face of extreme deception than a diploid population. Haplodiploids were consistently able to persist longer than diploids, and able to face higher densities of

deceptive orchids – even when males quickly became sperm depleted. The model predicted that when orchids elicit ejaculation from pollinators, they boost the production of sons from sperm-deprived female insects. Over several generations, this results in a stable male-biased pollinator population that can sustain both females' sperm needs for producing future generations, as well as the pollination of deceptive orchids. I confirmed this prediction via a survey of museum and digital records (Boakes et al., 2010), which we systematically assessed to reduce the bias inherent in such data (Chapter 6). Work on these records also revealed differences in active periods over a year for both males and females, as well as an estimated flowering period in *Cryptostylis* orchids.

In essence, *Cryptostylis* create more naïve pollinators for themselves by being deceptive enough to encourage sperm wastage. Not only would a male-biased sex ratio improve the chances of a female gaining sperm, but also the chance for the orchids to become pollinated. Building on the model that focused on pollinators, I showed that targeting haplodiploids might be the secret to the outstanding pollination success of *Cryptostylis* orchids (Gaskett and Herberstein, 2006; Gaskett et al., 2008; Schiestl, 2005): holding all else equal, orchids that target haplodiploid pollinators

consistently obtain higher pollination rates than those that target diploid pollinators (Chapter 7).

8.4 Individual impacts

In general, differences in behaviour and morphology among individuals of the same deceived species can arise as a result of changes in population dynamics (Eberhart-Phillips et al., 2018; Jiggins, Hurst and Majerus, 2000) or as direct responses to that cost (Feeney, 2017 ; Kilner and Langmore, 2011; Kokko and Mappes, 2013). In chapter three, I explored first whether there were any differences in pollinator behaviour and morphology for local populations that do or do not live with natural populations of sexually deceptive *Cryptostylis* orchids. Upon establishing that there may be differences, I assessed whether they could be a consequence of a male-biased sex ratio, or direct counter-adaptations, by correlating behavioural differences with the sole detected morphological difference: males from sites with orchids had significantly longer antennae than males from sites without.

There is little evidence to suggest that antennae length was a consequence of counter-adaptations. Instead, it seems that if deceit eliciting sperm wastage in haplodiploids increases the number of males in a population, we

might see more males competing for access to females (true or orchid). Selection may, therefore, act to produce males better suited for scramble competition – a behaviour observed in most solitary, parasitoid wasps (Quicke, 2014). I found evidence for traits associated with scramble competition, rather than discrimination between an orchid and a female. Faster first-male arrival time correlated with longer male antennae length. This may be due to more efficient searching, rather than discrimination. Although males from sites with orchids took longer to arrive, this may be due to males having more potential mates (either orchids or females) available to them in an area. Males' previous exposure to orchids did not impact the probability of pollination or ejaculation.

8.5 Caveats and future research

Field and lab experiments

Though this work quantifies an extreme cost of deception, further work is needed to test the potential for it to impact pollinators, and determine causation between orchid deception and changes in pollinators.

In terms of quantifying costs, successive mating of males with females (e.g. Boivin, Jacob and Damiens, 2005) would be an ideal way to measure an individual's tendency to become sperm limited. Moreover, we could assess

the actual costs of time wasted may through estimates of the flight and search times of male *L. excelsa*. With the advent of new tracking technologies, it may even be possible to track a male's day – and assess their time spent searching and visiting orchid patches (Ngo, Wu, Yang and Lin, 2019).

Intriguingly, the differences I observed between the local study populations may be a consequence of changes to their sex-ratio and enhanced scramble competition. Perhaps it is the interaction between haplodiploidy and sperm limitation that results in the success of sexually deceptive orchids that we perceive today (Gaskett, 2011). Alternatively, though I tried to make field sites as comparable as possible – habitat differences or landscape context differences might explain the differences observed between local populations.

However, as the models used in the analysis try to take these differences into account, the results I found in this thesis do at least suggest that there are changes in response to the presence of sexually deceptive orchids. Experiments that confirm this are an exciting new avenue for research – although they may be challenging to achieve.

As *L. excelsa* is difficult to rear in the lab, an easily reared ichneumonid (with similar life-history traits) might be a useful proxy for approaching

these questions. In the lab, experiments might simulate sexual deception by changing males' perceived abundance of females vs. competitors (e.g. through prolonged exposure to many females or many males before mating) and exploring differences in male behaviour and sperm use. Similarly, one might simulate sperm wastage by allowing males to mate with females but preventing sperm from entering the females' spermathecae (e.g. by using paper attached to males' abdomens to prevent copulation but allow excretion; Burke and Bonduriansky, 2019). Changes in male behaviour could be followed through time if this is carried out over several generations. These lab-based studies hold all other environmental factors constant, and so might allow for a causal understanding – rather than a correlation as presented here.

This proxy may also be useful for monitoring changes in sex-ratio of offspring as a result of sperm depletion. For instance, one could extract the proportions of sperm-depleted from my theoretical models presented here and impose this rate of unmated-ness on populations. In this way, we might test whether a persisting male-biased sex-ratio is realistic.

A full survey of males from sites with and without natural population of orchids, although difficult, would be ideal for determining whether males' behaviour and morphology is a consequence of counter-adaptations or the

male-biased sex ratio they are, potentially, experiencing. Such a survey could determine, in an unbiased way, what the sex ratio of local populations are; and whether there are morphological differences in males (or, indeed, in females). I attempted to ascertain sex-ratio at sites with and without natural populations of orchids using baited and unbaited sticky traps along transects at my field sites over six weeks. However, the results were not adequate for analysis (n= 7 *L. excelsa* caught across all six sites). I also reached out to owners of crops where *L. excelsa*'s larval host occurs, but the species had meagre emergence rates (estimated < 8%, through correspondence with Colin Tann), and are difficult to rear in the lab.

In general, cost-effective monitoring tools for parasitoid populations are lacking (Martinez et al., 2019). In this work, yellow sticky-traps proved insufficient for sampling the pollinator population. The development of improved traps (baited or with a chemical-based capture that does not rely on pheromones) would allow for an unbiased sample of the *L. excelsa* population. This would improve our understanding as to whether sexually deceptive orchids do, indeed, impact adult pollinator sex-ratios; whether pollinator abundance was comparable between sites; and whether the difference observed between local populations was only in males that do respond to orchids.

Modelling

My models here are a good, simple first step to explore the persistence of costly relationships between diploid and haplodiploid populations.

However, it holds all other aspects of the pollinators constant – the pollinator populations are wasps that are diploid or haplodiploid. Future work should explore the persistence of different sexually deceived pollinators. This has been explored somewhat by a Masters student I co-supervised in the course of my PhD (Kai-Fong et al., 2019): finding that thynnine wasps and solitary bees (both haplodiploid) have improved persistence over beetles and flies (both diploid). I hope to make these models available upon publication, so that other pollinator profiles can be tested.

Fungus gnats are the only other known sexually deceived pollinator to waste their sperm on orchids (Blanco and Barboza, 2005). Fungus gnats are not haplodiploid, but instead become more male-biased at cool temperatures when their deceiver, *Lepanthes* orchids, flower. A model that tracks changes in temperature and resulting sex-ratio in pollinator populations, as well as persistence in the face of deception might show whether temperature-mediated sex allocation acts as a *resilience* trait – much like haplodiploidy does in the case of *Cryptostylis* orchids.

8.6 A mechanism for persistence

Across these chapters, I have shown that these orchids impose severe costs on their pollinators that might, under other conditions, lead to their extinction: but mechanisms exist that might explain the persistence of the relationship.

I first introduced three mechanisms that could explain persistence: indirect fitness benefits, counter-adaptation and low encounter rates. Indirect fitness benefits likely do not maintain persistence in the case of extreme sexual deceivers, like *Cryptostylis* – as they do not offer any reward (Gaskett, 2011). Similarly, while counter-adaptations at the individual level may arise in response to deception, my results in this thesis indicate that any counter-adaptations are not sufficient to allow individuals to avoid exploitation through either evolved resistance (e.g. longer antennae associated with improved discrimination) or tolerance (e.g. larger testes or more sperm).

In my theoretical exploration of this problem, encounter rates were important in maintaining costly relationships. The antagonistic relationship between orchid and pollinator was best maintained when males rarely encountered sexually deceptive orchids. Low encounter rates

did not, however, result in higher pollination rates as observed in *Cryptostylis* orchids (Gaskett, 2011; Schiestl, Peakall, and Mant 2004). Instead, pollinator life-history (in this case, haplodiploidy) drove an increase in pollination rate and exploiter fitness.

As such, I propose that the persistence of the relationship between the extreme deceivers, *Cryptostylis*, may at least in part be explained by *Cryptostylis* orchids targeting haplodiploid pollinators. The exploitation of haplodiploid pollinators while eliciting ejaculation, specifically, allows orchids to prevent females in the local pollinator pool from being mated. This, in turn, creates more male pollinators that improve the orchids fitness and maintain the pollinator population by keeping some females mated. I present haplodiploidy as a first *resilience trait*: a life-history trait that simultaneously buffers the costs of deception, weakens counter-adaptations that would otherwise allow escape from deception, and improves exploiter fitness. In combination with other mechanisms of persistence, particularly low encounter rates, this hypothesis seems to be the most compelling for maintaining costly relationships while allowing deceivers to maintain high fitness over evolutionary time.

8.7 Concluding remarks

The dynamic interactions between orchids and their dupes are an excellent tool for investigating antagonistic relationships. By considering a pollinator perspective, I highlight that pollinator behaviour and life-history play important roles in understanding the persistence of costly, sexually deceptive relationships.

This novel approach could be useful for understanding other antagonistic coevolutionary relationships – do hosts of parasites have inherent traits that bring them resilience (Gilman, Nuismer and Jhwueng, 2012)? Do the prey of deceptive predators (O’Hanlon, Holwell and Herberstein, 2014)?

This research makes a significant contribution to this emergent idea by combining field-based research with mathematical modelling, and surveys of occurrence records. In general, I hope the findings of this thesis show the importance of taking alternative approaches and perspectives when seeking to understand evolutionary dynamics. And to you, the person reading this – most likely, you’re a PhD student or an orchid lover! – I hope you find as much resilience as these small, orange wasps.



Cryptostylis subulata and two sexually deceived pollinators, *Lissopimpla excelsa*, demonstrating the size difference apparent between males. Photo by Callum Young. Macquarie, Sydney, New Zealand.

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