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REPRODUCTIVE COMMUNICATION IN THE AUSTRALIAN GUMLEAF SKELETONIZER, *URABA LUGENS* (WALKER)

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THESIS ABSTRACT

Theoretical and empirical studies demonstrate that female moths vary their investment into pheromone-releasing behaviour according to both biotic factors (including age, mating status and competitive signalling) and abiotic factors (including temperature, host plant quality, and photoperiod). This suggests that female moths are capable of sophisticated strategic adjustment of their pheromone production, yet the impact of variation in pheromone output on male arrival rates and male preferences in moths are relatively unexplored. Furthermore, studies documenting sexual chemical communication in moths have focused primarily on the chemical nature of the signal, and have largely ignored female signalling strategies or chemical receiving structures (antennae), despite the costs of signalling and of maintaining signal receiving structures.

In Chapter 1, I provide a synthetic review of the results of my research on the gumleaf skeletonizer moth, *Uraba lugens,* in the context of the broader literature, and argue that while females strategically adjust their sex pheromone signalling behaviour and hence their attractiveness, males strategically balance their investment between longevity and antennal morphology, which reflects their mate searching capacity. In the context of chemical communication in moths, these females calling (pheromone-releasing) strategies reduce the likelihood of mating failure and allow males to maximise their encounter rates with females.

In Chapter 2, I explored how adult age influences the calling behaviour of virgin female *U. lugens* over four continuous ten-hour scotophases (dark periods). I found that female *U. lugens* alter their calling behaviour with age, but in contrast with theoretical predictions and empirical observations in other species, older females were less likely to call and spent less time calling than younger females. Older females, however, commenced calling earlier in the scotophase, suggesting a strategic shift, potentially to avoid competition from younger females. Behavioural assays with y-maze olfactometers showed that males prefer the pheromones produced by younger females, and that pheromone quality likely plays a role in this choice.

In Chapter 3, I explored how juvenile population density influences pheromone output in female *U. lugens*. I found that female *U. lugens* facultatively adjust their calling behaviour in response to socio-sexual cues: females that eclosed from high juvenile population densities

started calling earlier and spent more time calling than individuals eclosed from low juvenile population densities. Juvenile density also affected female pheromonal attractiveness: males prefer the pheromones produced by females reared at high juvenile densities. Females are likely to benefit from this strategic investment: increased investment into chemical signalling (at high densities) suggests that females compete with conspecific neighbouring signallers in order to avoid mating failure.

In Chapter 4, I explore how juvenile diet influences reproductive investment of both female (the quality of female sex pheromone) and male (pre- and post-copulatory) adults. I found that the effect of juvenile diet on adult fitness depended upon adult sex: in females, diet influenced body size, while in males diet influenced longevity. Juvenile diet also affected female pheromonal attractiveness: males tended to prefer the pheromones produced by females reared on host plants which has been supplemented with a fertiliser. Finally, host plant species affected male pre-copulatory investment: males reared on *Eucalyptus camaldulensis* have longer antennae but less dense sensilla than when reared on a different Eucalypt species, although there was no difference in the testes size of males reared on the two different species.

In Chapter 5, I explore how upregulation of immunity affects male antennal functional morphology, female pheromone quality, and other life-history traits. I found that immune activation affected male, but not female signalling investment: immune challenged males had a lower density of sensilla on their antennae, but female pheromonal attractiveness was not affected by their immune status. Nevertheless, immune activation reduced female investment into ovary mass, and the longevity of adult males and females increased following an immune challenge.

In conclusion, my different experiments consistently reveal that females alter their sex pheromone production and releasing behaviour for mate attraction in order to avoid mating failure, while males balance the resources allocated to longevity and chemoreception, which improves the likelihood of locating mates.

DECLARATION

This is to certify that:

(i) This thesis comprises only my original work towards my Ph.D.

(ii) Due acknowledgement has been made to all other material used

(iii) This thesis is fewer than 80, 000 words excluding references, tables and figures

Signed

Hieu Thi Pham

February 2020

PREFACE

This thesis is written as a series of stand-alone publications. These papers are either submitted or are in preparation for publication and there is thus some necessary repetition, in particular in the 'Methods' sections, between chapters. I have attempted, where possible, to reduce the overlap and instead highlight the key findings of this work.

I was the principal contributor and primary author of all chapters. My supervisors have been recognised for their invaluable contribution in all chapters and all other contributors are mentioned in the acknowledgements.

Manuscripts from this thesis:

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CHAPTER 1

Introduction

A significant, but often overlooked, challenge for dioecious insects is for members of the opposite sex to find each other. Drawing on sexual selection theory (see Bonduriansky, 2001; Trivers, 1972), it is widely assumed that while many males may never mate, this is an unlikely fate for females. And yet reproductive failure is not uncommon in natural populations of insects (Rhainds, 2010, 2019), and may select for virgin females to adopt strategic behaviour that optimises the balance between the quality of mate she attracts and the risk of remaining unmated.

Female insects utilize a range of signal modalities to advertise their location, such as the visual signals of fireflies (Stanger-Hall & Lloyd, 2015) and the auditory signals of crickets (Leonard & Hedrick, 2010). However, chemical signals are arguably the oldest and most widespread means by which females communicate their location (Wyatt, 2003) and are especially important among moths (Svensson, 1996). Female moths typically release sex pheromones from specialised glands, in minute quantities that can nonetheless travel long distances before being detected by potential mates and eliciting copulation (Wyatt, 2003). Since the first sex pheromone was identified in silk moths, *Bombyx mori*, by Butenandt et al. (1959), a very substantial research effort has resulted in an extensive catalogue of pheromone profiles of insects (Symonds & Elgar, 2008), especially for agricultural pests. However, the vast majority of these studies focus on providing details of the chemical composition of the sex pheromone, typically in the context of its use in controlling pest insect numbers (Howse et al., 1998).

Despite the wealth of information on pheromone synthesis and its function, there are still conspicuous gaps in our understanding of pheromone-releasing behaviours, especially the factors responsible for any variation in chemical signalling and their consequences for the intended receivers. In particular, it is unclear whether documented changes in signalling behaviour with age reflect physiological constraints or strategic investment (Umbers et al., 2015). My thesis examines the causes and consequences of variation in female signalling that reveals her location. My experimental approach aims to identify the impact of life history and ecological factors on both female signalling and male reception.

Studied species

The gumleaf skeletonizer moth, *Uraba lugens,* (Lepidoptera: Nolidae) (Fig. 1) is an ideal model species to examine chemical signalling from the perspective of both signaller and receiver. In

this species, adults eclose during late scotophase (dark period) or early in the morning (Campbell, 1962), and females commence calling a few hours after the onset of scotophase, with calling frequency peaking around seven hours after the onset of scotophase (Gibb et al., 2008). The adult lifespan of females is typically five to eight days, during which time they mate and oviposit, although the mating frequency of this species has not been quantified. Females have poor flight capacity (Harris, 1975), so their mating success depends critically on the attractiveness of their sex pheromones. Female mating rates under laboratory condition are low, and males can mate once only per night (personal observation). Thus, male fitness may be primarily determined by their longevity and their ability to locate receptive females quickly, rather than by postcopulatory selection mechanisms (see Simmons, 2001). Furthermore, various features of the life history of larvae of this species allowed me to explore the impacts of larval experience on adult signalling and reception. There is considerable variation in juvenile population density in this species: early-instar caterpillars are highly gregarious but they disperse at later developmental stages (forming a head-dress by the old head capsules) (Fig. 1) and live in smaller groups or become solitary (Campbell, 1962). U. *lugens* is phytophagous species that feeds on over 103 myrtaceous species, of which the genus *Eucalyptus* is dominant (Berndt & Allen, 2010). The species have been documented as a serious pest of numbers of *Eucalyptus* trees in Australia (Allen & Keller, 1991; Campbell, 1962; Cobbinah, 1983, 1985; Cobbinah et al., 1982; Farr, 1985, 2002; Farr et al., 2003; Morgan & Cobbinah, 1977; Potter & Stephens, 2005; Strelein, 1988) and New Zealand (Berndt & Allen, 2010; Gibb et al., 2008; Suckling et al., 2005). *U. lugens* is a capital breeding species that does not feed as an adult, and so the reproductive life-history parameters are determined by the resources obtained during the juvenile period. Nevertheless, whether these factors affect female investment into mate attraction and male investment in signal reception are not known, despite their obvious importance in shaping the mating biology of this species.

Figure 1. Gumleaf skeletonizer *Uraba lugens*: larvae with the head capsules attached on the head (left); adult (female). (Photo credit: Hieu Thi Pham).

Variation in female signals of location

The development of improved techniques of chemical analysis of pheromones has resulted in a wealth of information about the chemical composition of pheromones produced by different species (Symonds & Elgar, 2008). Surprisingly, intra-specific variation has been largely ignored, even though several studies document differences in both releasing behaviour and the chemical components of the pheromone. Theoretical and empirical studies indicate that females vary their investment into calling behaviour (pheromone-releasing behaviour) according to both biotic factors, including age, host plant quality, mating status and competitive signalling (Bjostad et al., 1980; Fiaboe et al., 2003; Gemeno & Haynes, 2002; Jacas & Peña, 2002; Lu et al., 2017; Ming et al., 2007; Noldus & Potting, 1990; Rehermann et al., 2016; Swier et al., 1977; Valles et al., 1992) and abiotic factors that include temperature, and photoperiod (Delisle, 1992; Delisle & McNeil, 1986, 1987a, 1987b; Jacas & Peña, 2002; Kamimura & Tatsuki, 1993; McNeil, 1991; Noldus & Potting, 1990). The variation in signalling investment can occur both between scotophases (Nascimento et al., 2016; Valles et al., 1992), and within the scotophase (Schal et al., 1987). In addition to variation in signalling behaviour, several studies reveal variation in the concentration and the component composition of pheromones either stored in the gland or released into the environment (Bjostad et al., 1980; Schal et al., 1987; Valles et al., 1992).

Understanding how age affects female signalling is important, since all females age, regardless of differences in demography, mating system and behaviour. The effect of female age on lepidopteran signalling has been examined in a range of species, primarily in the context of signalling investment by virgin females and the risk of reproductive failure (Delisle, 1995;

Mazor & Dunkelblum, 2005; Turgeon & McNeil, 1982; Webster & Cardré, 1982). Age may affect signalling in several ways, including 1) the likelihood she commences calling, 2) the duration of calling, 3) the timing of the onset of calling, and 4) the quantity and/or concentration of the pheromone plume. Umbers et al. (2015) demonstrated theoretically that virgin female moths should increase their duration of calling as they age in order to influence the arrival rates of males. In contrast, the calling investment (likelihood of calling and time spent calling) of female *U. lugens* decreased significantly with age (Chapter 2). It is possible that there are limited resources available for pheromone production in *U. lugens*. Nevertheless, I found that older females of *U. lugens* commenced calling earlier in the scotophase, suggesting a strategic shift that may reduce competition from younger females. This change in the latency to call in each scotophase may decrease the likelihood of reproductive failure for older females (Delisle, 1995; Mazor & Dunkelblum, 2005; Turgeon & McNeil, 1982; Webster & Cardré, 1982).

Theory also predicts that younger virgin females should release a lower pheromone concentration compared with older females (Umbers et al., 2015; Webster & Cardré, 1982). This can reduce nutrient expenditure associated with behavioural and chemical components of pheromone production, but may also be an example of indirect female choice for males with more sensitive antennae (Elgar et al., 2018; Johnson et al., 2017b). I show in Chapter 2, that males of *U. lugens* did not prefer the pheromones produced from multiple females over that of individual females (irrespective of female age), suggesting that pheromone concentration is not necessarily important for mate choice, although it appears to be for mate detection (Johnson et al., 2017b). Rather, males clearly preferred the pheromone produced by young over old females (Chapter 2). This suggests that the pheromones emitted by young and old females are qualitatively different, perhaps comprising different ratios of their chemical components. This could occur if aging females cease producing some pheromone components that are important for mate attraction.

Larval environment impacts on female signalling

Adult phenotype depends, in part, on larval experience, and there is emerging evidence of facultative adjustment of mating effort investment that is anticipated through the juvenile environment, including population density (Kasumovic & Brooks, 2011; Kokko & Rankin, 2006); diet (Boggs, 2009; Carsten-Conner et al., 2010; Darragh et al., 2019) and immune challenge

(Barthel et al., 2015; McNamara et al., 2013a). Most of these studies have focused on how these factors affect male, rather than female mating strategies (Kasumovic & Brooks, 2011).

Nevertheless, females are expected to adjust their investment into pheromone production and release in response to their juvenile environment. These adjustments may be manifested through variation in the timing and duration of calling and the quantity and/or concentration of the pheromone plume. For example, juvenile population density might indicate adult population density, which female moths can anticipate and adjust their reproductive investment accordingly. These adjustments may reflect Allee effects (Fauvergue, 2013; Rhainds, 2019) or competition with other females (Holdcraft et al., 2016; Lim & Greenfield, 2007). Allee effects predict that at lower population densities, females should increase calling effort, while mating competition effects predict that should be the case at higher population density. My experiments described in Chapter 3 reveal that the latter is more important in *U. lugens*: females that eclosed from high juvenile population densities commenced calling earlier in the first scotophase and typically called for a longer time. This adjustment suggests that females increase their signalling effort to avoid mating failure through competition from neighbouring females, which arises because males do not mate more than once during the scotophase. There are benefits to increasing investment in calling with higher adult densities of signallers (Evenden et al., 2015; Gascoigne et al., 2009; Lim & Greenfield, 2007; Lim et al., 2007; Palaniswamy & Seabrook, 1985; Regniere et al., 2013) and this behaviour is common in moths (Harari et al., 2011; Palaniswamy & Seabrook, 1985; Rehermann et al., 2016; Sadek et al., 2012; Stelinski et al., 2006).

Juvenile environment can also affect the quality and/or quantity of the sex pheromone produced by females and hence their attractiveness. Male *U. lugens* prefer the sex pheromone produced by females eclosed from high population densities (Chapter 3) and females that were reared on a high-nutrient diet (Chapter 4). Determining the nature of the differences in these pheromones was beyond the scope of this study, but field experiments reveal that pheromone concentration affects the type, but not number of attracted males (Johnson et al., 2017b). Evidence from diverse taxa indicates that changes in diet can result in both qualitative and quantitative differences in pheromones (Henneken et al., 2017; Henneken & Jones, 2017; Henneken et al., 2015). My behavioural assays showed that adult males of *U. lugens* tended to prefer the odours derived from females raised on a diet with a higher nitrogen content (Chapter 4). This male preference is consistent with the view that pheromones can provide

receivers with information about the quality of the signaller (Henneken et al., 2017): in this case, males male may benefit from mating with females reared on a high-quality, nitrogen-rich diet because they may produce more, and better nourished eggs (Xie et al., 2015). However, the impact of juvenile experience on the signalling investment of females is likely to be species-specific, simply because the chemical composition of pheromones differs between species and diet may not similarly affect the chemical components that comprise each pheromone. Additionally, while males showed preferences for the odours of females raised on nitrogen-rich diets, they were indifferent to odours of females raised on different *Eucalyptus* host plant species, despite the differences in carbon content (Chapter 4).

Larval environment and male reproductive investment

Male investment in reproduction can occur before or after copulation: the former includes traits that maximise mating opportunities (Andersson, 1994), and the latter includes mechanisms of avoiding or mitigating against the risk of sperm competition (see Simmons, 2001). Both traits are costly and, in some species, there may be a trade-off between pre-copulatory and post-copulatory reproductive investment (Simmons et al., 2017).

While the causes and consequences of male signalling and sperm competition are well understood in insects (Simmons, 2001), far less attention has focussed on how how signals revealing mate location shape the morphology of signal reception organs, such as antennae . For male moths, reproductive success depends upon locating signalling females, whose pheromones are detected by receptors on the antennae, a structure that is costly to produce and maintain (R. F. Chapman, 1982; Elgar et al., 2019; Elgar et al., 2018; Symonds et al., 2012). Remarkably, male *U. lugens* increase their investment in antennal size when reared at a low juvenile population density (Johnson et al., 2017a) thereby allowing them to be more able to locate younger females (Johnson et al., 2017b). I showed that juvenile diet also effects male investment on antennae: males of *U. lugens* that were reared on carbon-rich *Eucalyptus moorei* had longer antennae than males reared on *Eucalyptus camaldulensis* (Chapter 4). Additionally, a richer larval diet increases male adult longevity, which further improves their rate of mating success.

The surface of insect antennae support different types and numbers of sensilla (R. F. Chapman, 1982; Elgar et al., 2019; Zacharuk, 1985). The surface of these sensilla, such as tricodea sensilla, have a number of pores that function as chemical receiver micro-organs

(Hansson, 1995; Shields, 2005; Triseleva & Safonkin, 2006). These structures require significant and costly neural innervation (Niven & Laughlin, 2008; Sanes & Hildebrand, 1976; Stockl et al., 2016), and thus there may be a trade-off between antennae length and sensilla density. For example, males of *U. lugens* that were reared as larvae on carbon-rich host plants had longer antennae but lower sensilla density than males reared on host plants with lower carbon content (Chapter 4). This suggests that while a diet with a higher carbon content allows males to increase the size of the antennae, the costs of neural activities (Niven & Laughlin, 2008; Stockl et al., 2016) prevent investment in larger numbers of sensilla. Similarly, while juvenile immune challenges on male larvae of *U. lugens* did not affect the length of adult antennae, this challenge did reduce the density of sensilla (Chapter 5). A lower density of antennal sensilla is likely to compromise their capacity for pheromone detection and thus mate searching success (Elgar et al., 2019; Gill et al., 2013; Jayaweera & Barry, 2017; Johnson et al., 2017b).

Adult longevity is also important for male mate searching success in this and other species of lepidopterans (Barthel et al., 2015), and the larval environment may also affect adult longevity. Interestingly, I found some evidence that a reduced male investment into the detection of female pheromones may be balanced by increased longevity. For example, males reared on carbon-poor host plants have a lower density of sensilla but live longer than males reared on carbon-rich host plants (Chapter 4). Similarly, males that received a high dose of a non-pathogenic immune elicitor had a lower density of sensilla but a longer adult lifespan than males that received a low dose (Chapter 5). These data suggest that males with a poorer capacity for pheromone detection, nonetheless, have more time for mate searching.

While the biochemical costs of producing these pheromones is widely considered to be low (Alberts, 1992; Cardé & Baker, 1984; Fromhage et al., 2016; Kokko & Wong, 2007), my experiments reveal a pattern of variation in sex pheromone production and releasing behaviour that is consistent with a significant cost to chemical signalling. Harari et al. (2011) suggested that these costs, including energetic costs (Foster & Johnson, 2011) may have a negative impact on survival and fecundity. Additionally, signallers face the risk of attracting eavesdropping predators (Branco et al., 2006; Hendrichs & Hendrichs, 1998; Millar et al., 2001; Tinzaara et al., 2005). If there is no cost to chemical signalling, females would not be expected to alter the likelihood, timing of commencement, and the duration of calling. In the absence of strategic female signalling, we would not expect males to strategically allocate resources between chemoreception, longevity and post-copulatory reproductive investment. The

experiments described in my thesis provide compelling evidence of strategic olfactory signalling by female gum-leaf skeletonizer moths, *U. lugens*, a pattern that seems likely in other moths.

CHAPTER 2

Age-dependent chemical signalling in gumleaf skeletonizer moths,

Uraba lugens

ABSTRACT

Theoretical models predict that female moths should strategically adjust their signalling investment as they age, with older, virgin females increasing their pheromone output to attract males. I explored how adult age influences the 'calling' (pheromone-releasing) behaviour of virgin female gum leaf skeletonizer moths, *Uraba lugens*, over four continuous ten-hour scotophases (dark periods). Females commence calling shortly after eclosion, and for several hours into the scotophase. I found that female *U. lugens* alter their calling behaviour with age: but in contrast with theoretical predictions and empirical observations in other species, older females were less likely to call and spent less time calling than younger females. Older females, however, commenced calling earlier in the scotophase, suggesting a strategic shift, potentially to avoid competition from younger females. I also examined male olfactory preferences for pheromones from females of different ages. Y-maze assays showed that males prefer the pheromones produced by younger females, and that pheromone quality likely plays a role in this choice. The results of my experiments support the view that females can adjust their calling behaviour to attract particular males and provides insights into the response of males toward these different signallers.

INTRODUCTION

A significant, but often overlooked, challenge for dioecious species is for members of the opposite sex to find each other. It is often assumed that while many males may never mate, this is an unlikely fate for females, and yet reproductive failure is not uncommon in natural populations of insects (Rhainds, 2010, 2019). Female insects utilize a range of signal modalities to advertise their location, including the visual signals of fireflies (Stanger-Hall & Lloyd, 2015) and the auditory signals of crickets (Leonard & Hedrick, 2010). However, pheromones in the form of volatile chemicals are arguably the oldest and most widespread means by which females communicate their location (Wyatt, 2003). While a diversity of insects utilise sex pheromones (Baker, 1989; Harari & Steinitz, 2013; Steiger & Stökl, 2014), arguably the most widely studied are those of moths (Harari et al., 2011; Symonds & Elgar, 2008; Umbers et al., 2015). Chemical communication is especially important among moths, as their nocturnal lifestyle places less reliance on visual cues, unlike butterflies (Svensson, 1996). Female moths typically release sex pheromones from specialised glands, in minute quantities that can

nonetheless travel long distances before being detected by potential mates and eliciting copulation (Wyatt, 2003). While the biochemical costs of producing these pheromones is considered to be low (Alberts, 1992; Cardé & Baker, 1984; Fromhage et al., 2016; Kokko & Wong, 2007), there are costs associated with calling behaviour that may have a negative impact on survival and fecundity (Harari et al., 2011), including energetic costs (Foster & Johnson, 2011), and the risk of attracting eavesdropping predators (Branco et al., 2006; Hendrichs & Hendrichs, 1998; Millar et al., 2001; Tinzaara et al., 2005).

Sex pheromones convey more than simply the location of the signaller. They can provide a reliable means of advertising the condition or quality of the signaller to the receiver (Harari et al., 2011; Johansson & Jones, 2007; Svensson, 1996; Symonds et al., 2012) and hence have a role in sexual selection through mate choice (Davie et al., 2010; Steiger & Stökl, 2014). Johansson and Jones (2007) suggest that pheromones can be efficient signals for mate assessment, but compared with visual and acoustic modalities, surprisingly few studies have demonstrated a link between chemical signalling and sexual selection (Davie et al., 2010; Steiger & Stökl, 2014). Studies of moths have focussed on the role of female sex pheromones for male mate choice. This apparent sex-role reversal in sexual signalling is thought to arise because male moths experience significant costs in finding a mate (Fromhage et al., 2016; Kokko & Wong, 2007) but see Elgar et al. (2019), and they invest heavily in resource-expensive spermatophores (Dewsbury, 1982; Esfandi et al., 2015; Stockley, 1977; Wedell et al., 2002; Xu & Wang, 2014). Thus, males should be choosy (Edward & Chapman, 2011), as they will benefit from mating with females of a higher reproductive value, which may be affected by female weight (Callado-Galindo et al., 2013; Jaffe et al., 2007; Xu & Wang, 2009), age (Callado-Galindo et al., 2013; Liu et al., 2014; Xu & Wang, 2009), size (Rhainds et al., 1995; van Dongen et al., 1998) and previous mating experience (Callado-Galindo et al., 2013; Thomas, 2011).

Female reproductive failure (Rhainds, 2010, 2019) may select for virgin females to adopt strategic behaviour that maximises the quality of mate she attracts, while reducing the risk of remaining unmated as she ages. Theoretical and empirical studies demonstrate that females vary their investment into calling behaviour according to both biotic factors (including age, mating status and competitive signalling (Bjostad et al., 1980; Fiaboe et al., 2003; Gemeno & Haynes, 2002; Jacas & Peña, 2002; Lu et al., 2017; Ming et al., 2007; Noldus & Potting, 1990; Rehermann et al., 2016; Swier et al., 1977; Valles et al., 1992) and abiotic factors (including temperature, host plant quality, and photoperiod (Delisle, 1992; Delisle & McNeil, 1986,

1987a, 1987b; Jacas & Peña, 2002; Kamimura & Tatsuki, 1993; McNeil, 1991; Noldus & Potting, 1990). The variation in signalling investment can occur both across scotophases (Nascimento et al., 2016; Valles et al., 1992), and within the scotophase (Schal et al., 1987). This suggests that female moths are capable of sophisticated strategic adjustment of their pheromone production, yet the impact of variation in pheromone output on male arrival rates and male preferences in moths are relatively unexplored.

The effect of female age on lepidopteran signalling has been examined in a range of species, primarily in the context of virgin-female signalling investment and the risk of reproductive failure (Delisle, 1995; Mazor & Dunkelblum, 2005; Turgeon & McNeil, 1982; Webster & Cardré, 1982). An understanding of how age affects female signalling is important, given that all females age, regardless of differences in demography, mating system and behaviour. Umbers et al. (2015) demonstrated theoretically that virgin female moths should increase their signalling effort as they age in order to influence the arrival rates of males. Even with the costs associated with pheromone production considered low, their mathematical model demonstrated the potential for virgin females to adjust facultatively their pheromone investment. They proposed that this would allow females to select initially for high-quality males capable of detecting small amount of pheromones (Greenfield, 1981; Johnson et al., 2017b), and also to minimise the risk of failing to mate. Their review of the literature revealed that in the vast majority of studies of moth calling behaviour (23 of 32), females increased the time spent calling with age.

Umbers et al. (2015) further highlighted the lack of studies that provide a comprehensive link between female calling behaviour, realised pheromone titre and the functional male preferences for these traits. While the effect of female age on calling and pheromone titre has been established in a variety of moth species (Fiaboe et al., 2003; Jacas & Peña, 2002; Kanno, 1979; Lu et al., 2017; Mason & Johnson, 1989; Nascimento et al., 2016; Noldus & Potting, 1990; Webster & Cardré, 1982), the effect of these traits on functional male mating preferences and behavioural responses is rarely considered (Gemeno & Haynes, 2002; Johansson & Jones, 2007; Umbers et al., 2015). Previous studies have typically assumed that the quantity of pheromone in the female's gland reflects the quantity released into the environment and thus detectable by the male. However, studies investigating both pheromone gland titer and pheromone output showed that this is not always the case for moths. For example, the amount of pheromone emitted by female pickleworm moths, *Diaphania nitidalis*,

sharply increased and reached a peak after 7 hours of scotophase, while the concentration of pheromone in their gland remained consistent over the same time (Valles et al., 1992). Similarly, Schal et al. (1987) found that the pheromone release rate of female arctiid moths, *Holomelina lamae*, peaked at the beginning of calling and then sharply decreased, yet the titre of the gland remained constant through the scotophase. Ideally, measures of signalling investment should include the quantity of sex pheromone in the gland, the amount released into the environment and the frequency and duration of calling behaviour (Symonds et al., 2012; Umbers et al., 2015). However, volatile chemical signals, such as those used by lepidopterans, are typically released in low concentrations (Greenfield, 1981; Johnson et al., 2017b; Symonds et al., 2012) and degrade relatively quickly in the environment, making quantification of emissions difficult. Very few studies of Lepidoptera have quantified the emission of sex pheromones (Umbers et al., 2015) and those that have indicate considerable variation between individuals (Bjostad et al., 1980; Schal et al., 1987; Valles et al., 1992). Ultimately, the true measure of the functional importance of variation in female pheromone titre or emission rate is determined by male mate attraction. Olfactometer assays are a powerful experimental tool to explore the effect of variation in female pheromone output on attractiveness, and thus fitness. Comprehensive experimental approaches that assess female calling, pheromone titre, and olfactory attractiveness are required if we are to understand the causes and consequences of variation in female chemical signalling.

I used the gumleaf skeletonizer *Uraba lugens* (Lepidoptera: Nolidae) to quantify the effect of age on signalling and chemical communication. In this species, adults eclose during late scotophase (dark period) or early in the morning (Campbell, 1962), and females commence calling a few hours after the onset of scotophase, with calling frequency peaking at seven hours after the onset of scotophase (Gibb et al., 2008). Adults typically live for five to eight days, during which time females mate and oviposit, although the mating frequency of this species has yet to be quantified. Several studies have documented the chemical ecology and natural history of *U. lugens* in Australia (its native range) (Allen & Keller, 1991; Campbell, 1962; Cobbinah, 1983, 1985; Cobbinah et al., 1982; Farr, 1985, 2002; Farr et al., 2003; Morgan & Cobbinah, 1977; Potter & Stephens, 2005; Strelein, 1988) and New Zealand (where it is an introduced pest)(Berndt & Allen, 2010; Gibb et al., 2008; Suckling et al., 2005). Most recently, several studies have explored the impact of demographic cues on male investment in mate location (Johnson et al., 2017a, 2017b). However, the factors affecting female investment into

mate attraction are not known, despite their obvious importance in shaping the mating biology of this species.

Here, I explored the impact of age on the variation in female calling behaviour and male mating preferences. First, I quantified natural variation in female calling behaviour over the major component of the female's fertile period. I then examined the functional impact of this variation on male mating or mate-attraction preferences, by testing male olfactory preferences for females of different ages.

MATERIALS AND METHODS

Insect Culturing

A stock laboratory population of *U. lugens* from eggs or first- to second-instar larvae collected from *Eucalyptus spp*. trees in Royal Park, Melbourne, Victoria was established. This population was cultured in an incubator under reverse-phase photoperiod 14L:10D light conditions at 25°C and approximately 70% humidity. The juvenile stages were maintained with 20 – 30 individuals in plastic containers (1 L) and fed with fresh, mature leaves of *Eucalyptus* spp., which were replaced every two days. Pupae were sexed by size (male pupae are smaller than female pupae), transferred to individual vials (40 x 60 mm, 120 ml) and maintained under the same environmental conditions as larvae.

Mean female mating frequency

The mating frequency of female *U. lugens* is not known in the wild. In order to quantify the mating frequency of this species under laboratory condition, 3–5 pairs of recently-eclosed male and female moths were placed in a large mating container (9 L) and allowed to mate *ad libitum*. I repeated this for a total of 30 containers. Each container held several small cuttings of *Eucalyptus* spp. branches, which acted as a mating and oviposition substrate. Females were removed from the container after several days, and then dissected in order to count the number of spermatophores within the female's bursa copulatrix. The female's spermatheca (sperm storage organ) was ruptured under the microscope to confirm that viable sperm had been transferred. In total, 109 females, haphazardly selected from across the 30 mating containers, were dissected to obtain an estimate of mean population mating frequency.

Age-dependent calling strategies of virgin females

I examined whether females adjust their investment in sex pheromone signalling by varying the time of onset and/or duration of calling behaviour. Female *U. lugens* typically eclose several hours before scotophase, and then commence calling shortly after their wings have expanded and continue for several hours into scotophase. Calling is unambiguous in *U. lugens* as the female adopts a posture that exposes her pheromone gland. Virgin females (n = 52), eclosed from isolated pupae, were placed individually in clear plastic containers (40 x 60 mm, 120 ml) and were recorded for their calling behaviour for 10-hour of scotophase on four consecutive days. I recorded on the hour, every hour over the 10-hour trial period, whether or not females adopted a calling posture. The observations were made under a red-filtered light to mimic the scotophase. I recorded, for each female, the time of each calling event, the number of calling bouts (consecutive one-hour blocks of calling), and the total number of one-hour blocks in which females were calling.

Male preference for pheromones from young or old females

The response of males of *U. lugens* to pheromones emitted by females of different ages was assessed using a glass y-tube olfactometer (Fig. 1). Here, a standardized, continuous air flow was introduced at the end of each arm of the y-maze, passing over the females and on to the receiving male. A single 'young' (≤ 36 hrs post eclosion) female was placed in one arm and a single 'old' female (3 - 4 days post eclosion) was placed in the other arm and left to acclimate for one hour. After both females commenced calling, a 'young' (≤ 36 hrs post eclosion) virgin male was introduced into the central arm of the y-maze, and was deemed to have responded when he either walked or flew toward the airflow, travelling at least 5 cm into one of the arms of the y-maze and remaining there for more than 1 min. If males did not make any movement after 30 mins or moved into an arm of the y-maze in less than 10 seconds (indicative of random male movement due to being placed in the olfactometer, rather than an active choice) they were excluded from further analysis and replaced with another male. Males were given 60 min to make a choice, and males that did not make a choice during this period were excluded from the analysis. Each of the male moths was used once only, and pairs of females were used for two trials only (with females never occupying the same arm of the olfactometer). Pairs of females were re-used on the same day, and then discarded. The olfactometer apparatus was washed with water and dried after each trial, and the position of the young and old females

was rotated each trial to remove positional effects. The trials were conducted during the middle of the scotophase, when moths are most active (personal observation).

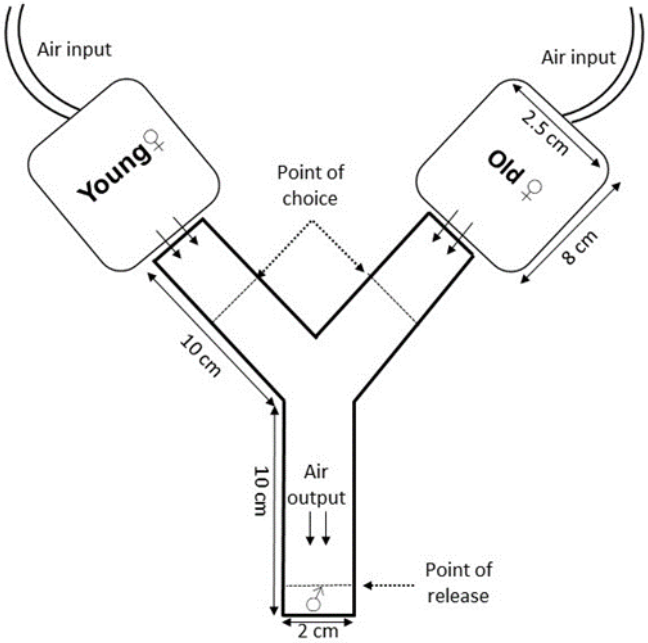

Figure 1. Schematic of the y-maze apparatus used to assess male preferences for sex pheromones from young and old females.

I conducted a second experiment to determine if male preference for young or old females is driven by quantitative or qualitative differences in female sex-pheromones production. Here, I followed the same methodology as above, but subjected males to four treatments that varied both the number and age of females that were placed in each of the arms of the y-maze olfactometer: one young vs two young females (n = 24); one old vs two old females (n = 21); one young vs two old females (n = 23); and two young vs one old female (n = 17). Each male moth was used once only, and pairs of treatment females were used for two trials only (again, females never occupied the same arm of the olfactometer). Male preference behaviours were recorded, as in the previous experiment.

Statistical Analysis

All the statistical analyses were conducted in R studio, version 3.5.2 (R_Core_Team, 2018). For my analyses of virgin female calling behaviour, I excluded from the data set females who did not survive the four consecutive scotophase observation periods (n = 12). I also excluded

females that eclosed > 5 hours after the onset of the first scotophase (n = 4), to ensure females were of comparable age. In total, 39 females were used in the analysis.

The effect of female age on the likelihood that a female called was explored using a chi-square test. Post-hoc differences in female age were analysed using paired chi-square comparisons, with significance levels adjusted using a sequential Bonferroni procedure to limit the Type 1 error rate. I was unable to analyse these data using a Generalized Linear Mixed Model, as there was no variation in female calling likelihood on day one.

The effect of female age on the time of onset of calling in each scotophase was analysed with a Generalized Linear Mixed model (package 'lme4') (Bates et al., 2015), with a Poisson distribution. Female identity was included as a random effect.

I then examined the total number of hours spent calling by a female each day. Not all females had eclosed for the entire first scotophase, therefore I expressed calling duration as a proportion of the time that females were eclosed and able to call each day, hereafter 'proportion of time calling'. The effect of female age on the proportion of time calling was analysed using a General Linear Mixed Model with a normal distribution, and female identity was incorporated as a random effect. Post-hoc tests (Tukey's HSD) were used in all models with significant effects of female age. Finally, the impact of female calling investment (proportion of time spent calling) and female body size on female adult longevity was explored using a General Linear model.

Male olfactory preference trials were analysed using chi-square tests. For every analysis, I examined if males exhibited a directional bias (left or right arm of the olfactometer). However, these were uniformly non-significant, and are not reported.

I also analysed the above male olfactory preference data to examine whether males exhibited a preference for female number *per se*, by comparing the data from trials when males chose between one young vs two young females and one old and two old females. I also analysed whether males exhibited a preference for young females, *per se*, by comparing the data from trials when males chose between one young vs two old females and one old and two young females. For these analyses, I used Generalized Linear Mixed Models, using a binomial error distribution, with female pair identity included as a random effect.

For all models, non-significant interaction terms were removed from final models. For all General Linear (Mixed) models, I optimally power transformed all dependent variables to maximize normality of residuals, and the exponents used were noted with every analysis.

RESULTS

Mean female mating frequency

The mating frequency of *U. lugens* moths in the laboratory condition was low, only 20 females of the 109 dissected having mated (18.34%). Of the 20 females that mated, the mean number ± standard error of spermatophores present was 1.15 ± 0.08.

Age-dependent calling strategies of virgin females

Investment in calling behaviour varied with both period of scotophase and female age. The proportion of calling females of any age increased during the scotophase, peaked at around 6 hours and declined sharply, with no females calling after 10 hours (Fig. 2).

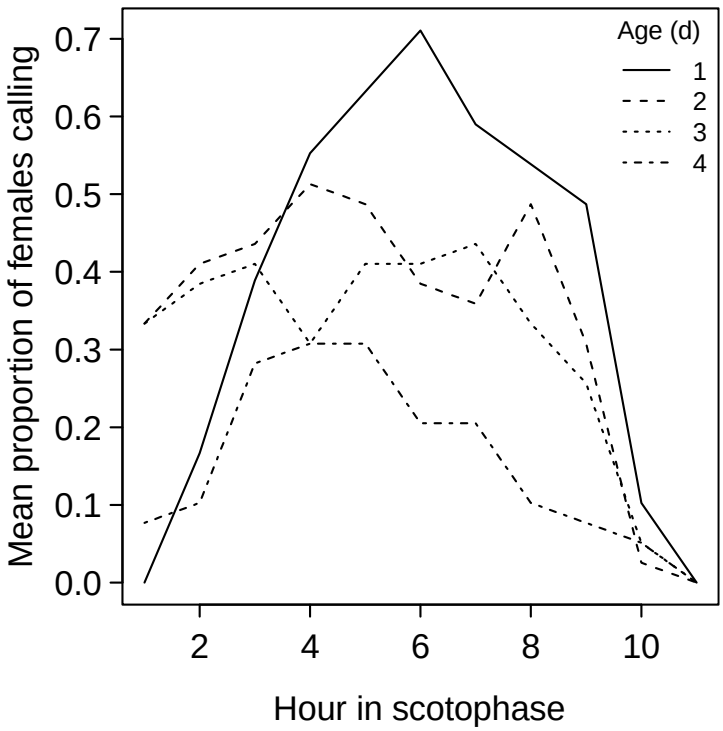

Figure 2. The mean proportion of virgin females calling (n = 39) at different ages (days) over the 10 hours scotophase

The likelihood of a female calling on any given day was affected by her age (χ^2 = 43.56, p < 0.001; Fig. 3). The percentage of one-day-old calling females was significantly higher than two and three-day-old calling females (χ^2 = 7.69, p = 0.005) and significantly higher than four-day-old calling females (χ^2 = 34.67, p < 0.001). The percentage of two and three-day-old calling females was significantly higher than four-day-old calling females (χ^2 = 15.47, p < 0.001).

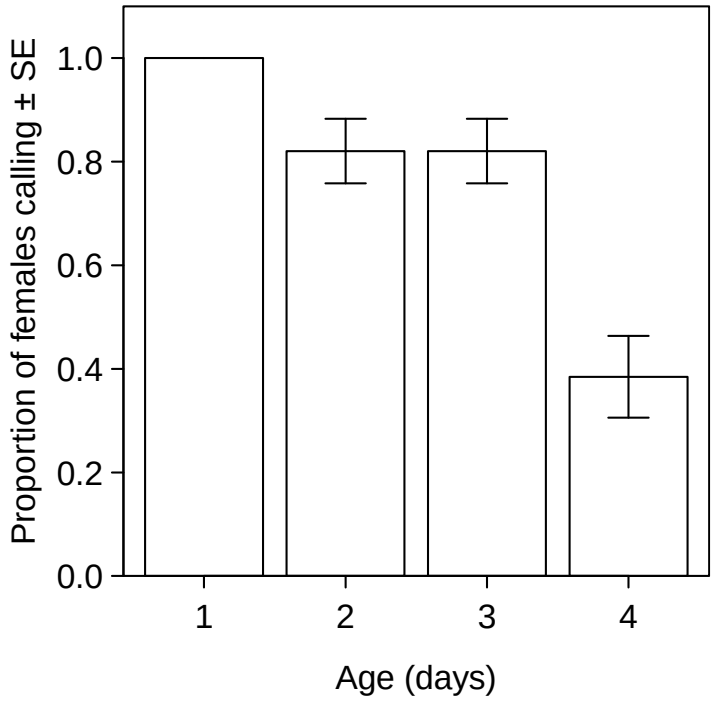

Figure 3. The proportion of female calling (±SE) (n = 39) at different ages (days).

The latency until calling within each scotophase (raised to the exponent 1.8) was affected by female age ($\chi^2 = 62.32$, $p < 0.001$). Post-hoc tests revealed that two and three-day-old females commenced calling significantly earlier in the scotophase than one-day-old females ($p < 0.001$). However, four-day-old females commenced calling later than three-day-old females ($p = 0.015$) (Fig. 4).

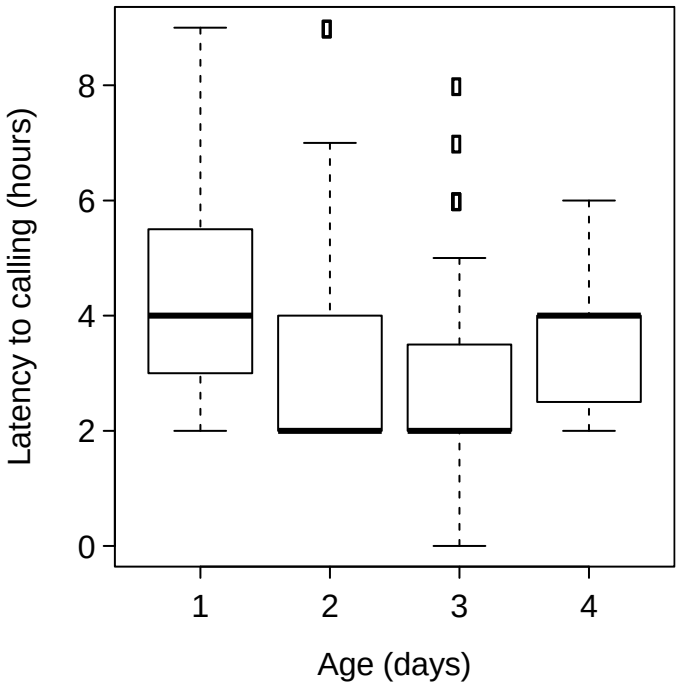

Figure 4. The median (and interquartile range) of latency to calling for females (n = 39) of different ages (days).

The proportion of the scotophase spent calling (raised to the exponent 0 .92) by females was significantly affected by female age (χ^2 = 50.14, p < 0.001). Post-hoc tests reveal that the proportion of time spent calling by three-day-old females was shorter than one-day-old females (p = 0.02) and four-day-old females was significantly shorter than younger females (p < 0.001) (Fig. 5).

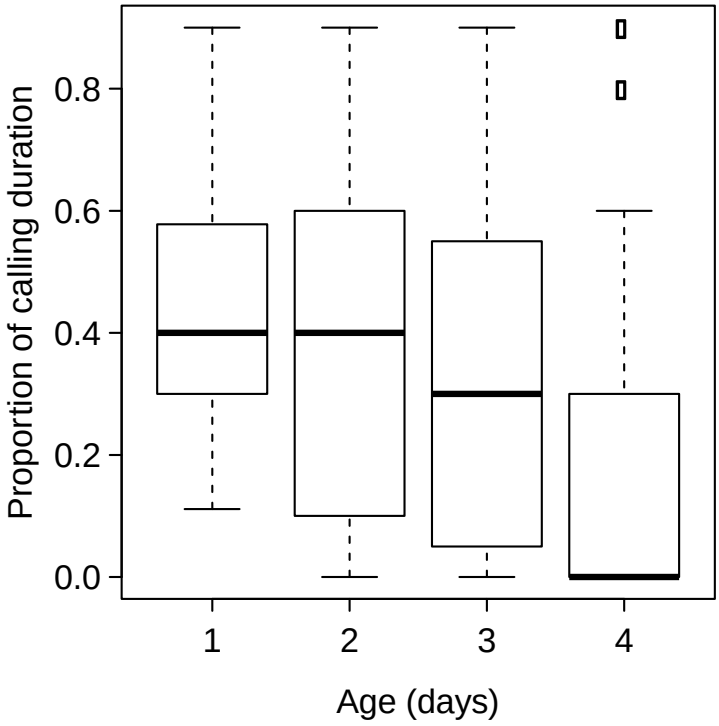

Figure 5. The median (and interquartile range) proportion of time calling by females (n = 39) at different ages (day).

Finally, female adult longevity was not affected by the total proportion of time she spent calling ($F_{1,25}$ = 0.00, p = 0.96) or her body size ($F_{1,25}$ = 0.49, p = 0.49). A non-significant interaction between time spent calling and body size was removed from the final model.

Male preferences for pheromones from young or old females

Male preferences for females of different ages and in different numbers are given in Fig. 6. Twenty-seven males (from 32 trials) made a successful choice between one young and one old female and showed a strong preference for pheromones emitted from young rather than old females (young = 23; old = 4; χ^2_1 = 13.37, p = 0.0003; Fig. 6). Seventeen males (from 22 trials) made a successful choice between two young females and one old female, and showed a strong preference for pheromones emitted from two young rather than one old female (two

young = 15; one old = 2; $\chi^2_1 = 9.94$, p = 0.002; Fig. 6). Of the 27 trials involving a choice between one young and two young females, 24 males made a successful choice but showed no preference for either set of females (one young = 12; two young = 12; $\chi^2_1 = 0.00$, p = 1.00; Fig. 6). Similarly, 21 males a successful choice (out of 25 trials) between one old female or two old females but showed no preference for either set of females (one old = 7; two old = 14; $\chi^2_1 = 2.33$, p = 0.13; Fig. 6). Finally, 23 males made a successful choice (out of 30 trials) between one young or two old females but showed no preference for either set of females (one young = 14; two old = 9, $\chi^2_1 = 1.09$, p = 0.30; Fig. 6).

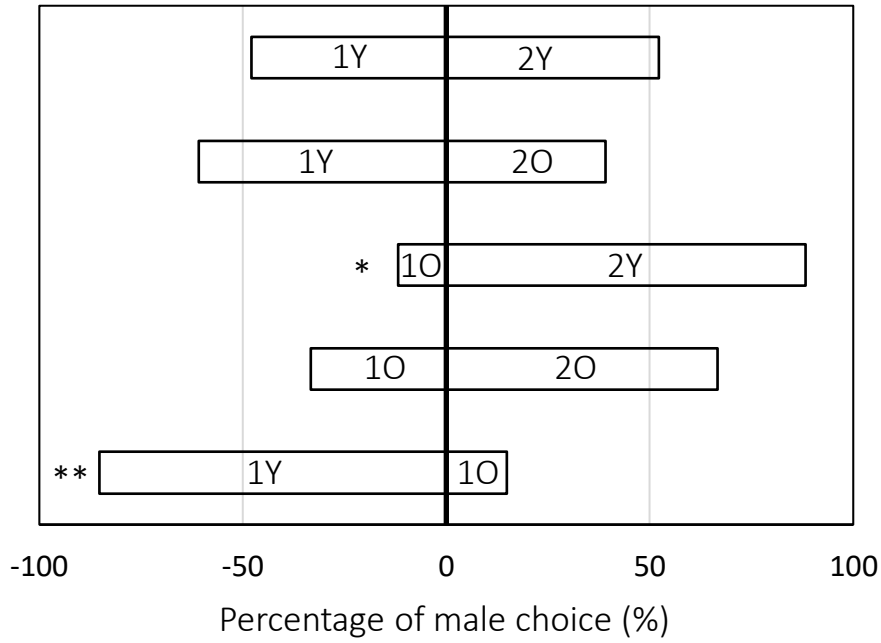

Figure 6. The proportion of males choosing between young (Y) or old (O) females, when females were present in different numbers (1 or 2). Asterisks indicate significant differences in male preferences (* = < 0.05; ** < 0.001).

I analysed the above male preference data to examine whether males exhibited a preference for female number *per se*, by comparing the data from trials where males chose between one young vs two young females and one old vs two old females. I also analysed whether males exhibited a preference for young females, *per se*, by comparing the data from trials where males chose between one young vs two old females and one old vs two young females. Males significantly prefer young females, regardless of the number of females of either treatment present (young females, n = 53; old females, n = 15; $\chi^2_1 = 20.43$, p < 0.001). Males did not

prefer the pheromone from multiple females compared with single females, regardless of their age (one female, n = 50; two females, n = 35; $\chi^2 = 2.65$, p = 0.10).

DISCUSSION

My data revealed that the calling behaviour of females of *U. lugens* varies with their age. The likelihood of calling was highest in one-day-old females and significantly reduced as females aged. I also found that older females called earlier in the scotophase, but for a shorter duration than younger females. Male olfactory preferences were also shaped by female age; with males exhibiting a strong preference for the pheromone from young females compared with older females. Together, these data reveal that females of *U. lugens* can adjust their behavioural investment into mate attraction, and that the qualitative and/or quantitative changes in female pheromone titer has implications for male mate choice.

Low mating rates are not unusual for moths in captivity, consistent with what I observed in *U. lugens*. However, Johnson et al. (2017b) suggested much higher mating rates in natural populations. The low mating rates in captivity may arise for several reasons: an absence of air flows that helps disperse pheromone and allows the males to detect 'plumes'; and/or that the air column within the containers becomes saturated with odour and thus offers no directional information about the source of the pheromone.

Investment into pheromone production is multi-faceted, and can be reflected in 1) the likelihood of a female commencing calling, 2) the duration of calling, 3) the timing of the onset of calling, and 4) the quantity and/or concentration of the pheromone plume. These patterns appear to be species-specific, and investment into these relative components of pheromone output may trade-off against each other (Umbers et al., 2015). The inter-specific patterns of when females commence calling are shaped by female life-history. For long-lived species, females typically commence calling several days after eclosion (as they reach reproductive maturity (Kanno, 1979), and the likelihood of females calling gradually increases, peaking in the middle of their adult lifespan, and reducing sharply with old age (Nascimento et al., 2016; Noldus & Potting, 1990; Turgeon & McNeil, 1982). However, signalling investment for short-lived species may be different, especially those species in which adults do not feed. These moths tend to start calling on the first day of emergence, with the proportion of females calling initially very high, and then reducing with age (Fiaboe et al., 2003; Webster & Cardré, 1982).

The low calling frequency of old females, such as in *U. lugens*, might reflect biochemical limitations of pheromone synthesis within her gland, the physiological limitations of old females to commence calling behaviour (Delisle & Royer, 1994; Mazor & Dunkelblum, 2005; Webster & Cardré, 1982) or simply physiological senescence (Foster & Johnson, 2011). Adult female *U. lugens* do not feed as adults so the available nutrients are fixed following eclosion, and must support a short adult lifespan (6-8 days). The fecundity of *U. lugens* is high (Farr, 2002), suggesting that most resources are directed to investment in eggs, perhaps at the expense of signalling.

Contrary to theoretical models and empirical studies (Umbers et al 2015), the calling investment of female *U. lugens* decreased significantly with age, and was lower even in three-day-old females. Umbers et al (2015) documented that most studies (23 of 32) of moth calling behaviour report that female calling increased with age. For example, female rice stem borer moths, *Chilo suppressalis,* increased their calling duration and length of calling bouts as they aged (Kanno, 1979). Why do females of *U. lugen*s not conform to this pattern? Reduced calling with age is not uncommon in moths: a comparable reduction in time spent calling has been recorded in the cabbage looper moth, *Trichoplusia ni,* (Bjostad et al., 1980), and the pink stem borer, *Sesamia calamistis* (Fiaboe et al., 2003), while in other species the time spent calling did not covary with female age (Jacas & Peña, 2002; Nascimento et al., 2016). Clearly, caution must be used when making generalisations about signal investment and age. Indeed, most of the studies reported by Umbers et al. (2005) did not observe female calling behaviour over her entire adult life span, but rather was confined to several days post eclosion. It is therefore possible that the observed increase in signalling effort with female age relates to a specific stage in her lifespan. It is possible that there are limited resources available for pheromone production in *U. lugens*, perhaps reflected in the cessation of pheromone production and release following mating. The cost of signalling is predicted to be greater for individuals in poorer condition (Johansson & Jones, 2007; Kotiaho, 2001a) or older females in species in which adults do not feed, such as *U. lugens*. Small females of the European grapevine moth, *Lobesia botrana,* that call more frequently lay fewer eggs than small females that call less frequently (Harari et al., 2011), and a reduction in calling with age in *U. lugens* may reflect a similar trade-off with fecundity.

Many studies show that older females tend to commence releasing pheromone earlier in the scotophase than younger females, regardless of their mating system (Nascimento et al.,

2016; Noldus & Potting, 1990). This change in the latency to call in each scotophase might allow older females to avoid competition with younger females, whose pheromone may be more attractive to males. Fromhage et al. (2016) suggested that selection may favour earlier female receptivity in order to extend the "mating window" and thereby increase their encounter rates with males, who are able to commence searching for a mate earlier in the scotophase. It can take several hours for newly-eclosed female *U. lugens* to be physiologically mature for calling. There are likely to be more searching males at the commencement of the scotophase because copulation can take up to four hours (personal observation) and thus males can typically mate only once per day. Furthermore, male *U. lugens* are likely to be polygynous, given that females can mate multiply and live from 8-13 days (personal observation), and so males that mate early in the scotophase have more time to replenish their sperm supply for the next copulation (Fromhage et al., 2016). Thus, older, virgin females that call earlier may decrease the likelihood of reproductive failure (Delisle, 1995; Mazor & Dunkelblum, 2005; Turgeon & McNeil, 1982; Webster & Cardré, 1982).

The changes in female calling behaviour are reflected in male olfactory preferences for odours from females of different ages. Males prefer the odour of younger females, suggesting that the quality or quantity of the pheromones emitted by different-aged females can be distinguished by the receiver. The benefits to males of detecting and preferring younger females is clear: young females have a greater residual reproductive value (Xu & Wang, 2009), given the correlation between female age and female fecundity and fertility rates (Foster & Howard, 1999). Furthermore, younger females are more likely to be virgin (Delisle, 1995) and so males can avoid a lower fertilisation rate through sperm competition by avoiding mating with older females.

What do male preferences for pheromones of females of different age and quantity tell us about age-dependent pheromone composition in this species? Theory predicts that younger virgin females should release a lower pheromone concentration compared with older females (Umbers et al., 2015; Webster & Cardré, 1982). This can reduce nutrient expenditure associated with behavioural and chemical components of pheromone production, but may also be an example of indirect female choice for males with the most sensitive antennae (Elgar et al., 2018; Johnson et al., 2017b). Indeed, males did not prefer the pheromones produced from multiple females over that of individual females (irrespective of female age), suggesting that pheromone concentration is not necessarily important for mate choice, although it

appears to be for mate detection (Johnson et al. 2017b). Rather, females clearly preferred the pheromones produced by young over old females. This suggests that the pheromones emitted by young and old females are qualitatively different, perhaps comprising different ratios of their chemical components. This could occur if females cease producing some pheromone components that are important for mate attraction as they age. For example, the quantities of two pheromone components, 2me-16c and n-17c, of *Holomelina lamae* increased with age but the amount of another component, 2me-19c, decreased with age (Schal et al., 1987). Changes in the quality of pheromone output with age might be a consequence of a decline in some, but not all, fatty-acid enzymes that are responsible for pheromone synthesis (Foster & Greenwood, 1997). Clearly, a quantitative analysis of the main pheromone components of female *U. lugens* as they age is required to elucidate the precise mechanism for the changes in female pheromonal attractiveness. Unfortunately, this is beyond the scope of this current investigation.

Taken together, the decline in female calling investment with age, and the clear male preference for younger females, suggest that the earlier onset of calling for older females may be a strategy to increase the likelihood of virgin females attracting a mate, especially if there is local female: female competition for arriving males. The outcome of the male olfactory preference trials revealed that older females are likely losers in the game of signalling competition. Earlier calling of older females may be an adaptive strategy to reduce the risk of reproductive failure (Rhainds, 2010, 2019), without altering the quality of their pheromone output, which typically declines with age in female moths. These results are consistent with the view that female pheromones are honest signals of female quality (Harari et al., 2011; Johansson & Jones, 2007), and further emphasize the role of sex pheromones in mate assessment.

CHAPTER 3

Socially-cued anticipatory adjustment of female signalling effort in gumleaf skeletonizer moths, *Uraba lugens*

ABSTRACT

Juvenile population density can have profound effects on subsequent adult development, morphology, and reproductive investment. Yet, little is known about how the juvenile environment affects adult investment into chemical sexual signalling, perhaps due to the historical assumption that pheromone production is not costly. In the gumleaf skeletonizer moths, *Uraba lugens,* males facultatively increase investment into antennae (pheromone receiving structures) when reared at low juvenile population densities. However, whether there is a comparable adjustment by females into pheromone investment has not been tested. I explored how juvenile population density influenced both the 'calling' (pheromone-releasing) behaviour of female *U. lugens*, and the quality of their pheromones they released. Juveniles were reared at low, medium and high population densities (one, five and 25 individuals/container, respectively). First, I found that juvenile population density affected adult body size, but not longevity. I also found that female *U. lugens* facultatively adjust their calling behaviour in response to socio-sexual cues: females that eclosed from a high population density started calling earlier and spent more time calling than individuals eclosed from low juvenile population densities. Juvenile density also affected female pheromonal attractiveness: y-maze assays showed that males prefer the pheromones produced by females reared at high juvenile densities. The results of my experiments support the view that females adjust their calling behaviour based on juvenile cues that anticipate the future socio-sexual environment. Females are likely to benefit from this strategic investment: increased investment into chemical signalling (at high densities) suggests that females compete with conspecific neighboring signallers in order to avoid mating failure.

INTRODUCTION

There is accumulating evidence of significant variation in female pheromone production in response to a range of biotic and abiotic factors (Delisle, 1992; Delisle & McNeil, 1986, 1987a, 1987b; Jacas & Peña, 2002; Kamimura & Tatsuki, 1993; McNeil, 1991; Noldus & Potting, 1990) suggesting there is a cost of female pheromone production. However, the conventional assumption is that the costs of pheromone production in moths is low (Alberts, 1992; Cardé & Baker, 1984; Fromhage et al., 2016; Kokko & Wong, 2007). Interestingly, females can adjust

strategically their investment into pheromone production and release according to socio-sexual cues (Rehermann et al., 2016; Sadek et al., 2012; Valles et al., 1992; Yang et al., 2009). In particular, recent studies have focused on plasticity in female calling (pheromone-releasing behaviour) in response to conspecific pheromones, referred to as pheromone autodetection (Holdcraft et al., 2016) or female pheromonal chorusing (Lim & Greenfield, 2007). Here, female moths adjust their signalling effort according to the presence or absence of their adult conspecifics. For example, female noctuid moths, *Pseudaletia adultera,* and females arctiid moths, *Utetheisa ornatrix,* called longer and more frequently when grouped with conspecific females compared with solitary females (Lim & Greenfield, 2007; Lim et al., 2007; Rehermann et al., 2016). In contrast, some species facultatively reduce their signalling investment in response to conspecific pheromones (Gokce et al., 2007; Harari et al., 2015; Yang et al., 2009). For example, the female armyworm, *Spodoptera exigua,* performed shorter calling bouts when exposed to conspecific calling neighbours (Yang et al., 2009). Conspecific presence can also affect the timing, rather than the duration of calling, with competitors inducing both earlier (Rehermann et al., 2016; Stelinski et al., 2006) and later (Sadek et al., 2012; Yang et al., 2009) onset within the same scotophase. Thus, female responses to adult socio-sexual cues are highly species-specific and may differentially affect various components of female calling behaviour.

There is emerging evidence of facultative adjustment of mating effort investment that is anticipated through the juvenile environment (Kasumovic & Brooks, 2011). In particular, population density has an important influence on both male and female mating strategies (Kokko & Rankin, 2006). In populations where density fluctuates between generations, selection is predicted to favour individuals that can assess cues that provide information on their future reproductive environment, adjusting their investment accordingly (Elgar et al., 2019; Kasumovic & Brooks, 2011; Kokko & Rankin, 2006). Male Pyralid moths use larval population density as a cue of future sperm competition risk, increasing their gametic investment when reared at high density (Bhavanam & Trewick, 2017; Gage, 1995; McNamara et al., 2010; McNamara & Simmons, 2017); and male gumleaf skeletonizer moths, *Uraba lugens,* increase their investment in antennal size when reared at low juvenile population density, presumably to increase their ability to detect sparsely-located mates (Johnson et al., 2017a).

In contrast, few studies ask whether females similarly anticipate future socio-sexual environment using cues in the juvenile environment and adjust their reproductive investment

accordingly. Like males, the resources required for female reproduction, which may include sex pheromones as well as eggs, are typically acquired during the juvenile stage of development (Gullan & Cranston, 2010), and especially so in capital breeders, where adults do not feed. Females must signal their location and receptivity for mating (Harari et al., 2011; Johansson & Jones, 2007; Svensson, 1996; Symonds et al., 2012). The rate of female mating failure is likely to be higher in insects where females are less mobile, and when populations have lower densities – "a mate-finding Allee effect" (Fauvergue, 2013; Rhainds, 2019). Thus, there are two alternative density-dependent mechanisms affecting female mating failure, generating different expectations regarding female calling strategies. First, mate-finding Alee effects predict that females from low-density populations should increase their signalling efforts by calling more frequently and/or releasing high quantity/quality of sex pheromones in order to increase the likelihood of attracting the sparsely-located mates. Similarly, females from high-density populations may also increase their signalling effort to avoid mating failure due to high signalling competition from neighbouring females. Nevertheless, facultative female adjustment in chemical communication investment in response to juvenile population density has been over looked, perhaps because it is widely assumed that pheromone production is not costly (Alberts, 1992; Cardé & Baker, 1984; Fromhage et al., 2016; Kokko & Wong, 2007). However, there is increasing evidence that female pheromone production is not only costly, but is also facultatively adjusted in response to the presence of conspecifics (Harari et al., 2015; Lim et al., 2007; Rehermann et al., 2016).

The gumleaf skeletonizer moth, *Uraba lugens,* (Lepidoptera: Nolidae) is an ideal model to examine the effect of juvenile population density on female signalling and chemical communication. Females have poor flight capacity (Harris, 1975), so their mating success depends largely on the attractiveness of their sex pheromones, and females adjust their calling behaviour in response to the risk of reproductive failure (Chapter 2). There is considerable variation in juvenile population density in this species. Females lay clusters of eggs, ranging in size from 20 to 500 eggs/cluster (Morgan & Cobbinah, 1977). Early-instar caterpillars are highly gregarious, eating, molting and moving as a group (Campbell, 1962). However, caterpillars disperse at later development stages and live in smaller groups or become solitary (Campbell, 1962). *U. lugens* is a capital breeder and adults do not feed or drink. Adult *U. lugens* eclose during late scotophase (dark period) or early in the morning (Campbell, 1962), and females commence calling a few hours after the onset of scotophase, with calling frequency peaking at

seven hours after the onset of scotophase (Gibb et al., 2008). Male *U. lugens* adjust their investment in reproductive strategies according to their juvenile environment: males reared at low population densities have larger wings and antennae (Johnson et al., 2017a, 2017b), the latter improving mate detection (Johnson et al., 2017a, 2017b).

It is not known whether females can detect cues in the juvenile environment and subsequently adjust their investment into mate attraction, through the timing and duration of calling and the quantity and/or concentration of the pheromone plume. Here, I explored the impact of juvenile population density on variation in female calling behaviour and female pheromonal attractiveness. First, I quantified natural juvenile population density using a series of field observations. Second, I reared caterpillars at low, medium or high juvenile population density that reflect natural population densities and observed subsequent female investment into sexual signalling over her fertile period. Finally, I examined the functional impact of this calling variation on female pheromonal attractiveness by testing male olfactory preferences for females eclosed from different population densities. I aimed to test whether females adjust their signalling investment according to cues obtained in their juvenile period, and whether these signalling strategies are predicted by mate finding Allee effects, or by local signalling competition.

MATERIALS AND METHODS

Field observations: Population demography of juvenile U. lugens

In order to understand how natural variation in population density might affect chemical communication in *U. lugens*, I noted the density of natural populations of caterpillars every two weeks at Royal Park, Melbourne, Victoria (37° 47′ 35″ S, 144° 57′ 16″ E) from late January to early April 2018, totalling 7 collection trips. Fifteen to twenty groups of juvenile-stage larvae were haphazardly chosen each collection trip. Individuals were collected from a maximum of 1.5m from the ground. Only one group per tree was collected. A group was defined as all of the individuals present on a single branch (from within 20cm of the distal tip of the branch). I was able to assign each larva to one of three age classes (‘young’, ‘middle’ and ‘old’) by noting the size and number of attached head capsules, because larvae older than the fifth instar retain each molted head capsule on their head as they progress to pupation (morphological descriptions of each age group described in Table 1, see also Low et al. (2016). In total, 123

groups of caterpillars (young age, n = 41; middle age, n = 32, and old age, n = 50) were recorded.

Table I. Identification of larval instar of *U. lugens* by body size and the presence of head capsules in the field survey

Larval instar	Morphological and behavioural identification
1st - 4th ('young')	Small body size (length range: 0.21 to 3.98 mm). No previously molted head capsules present. Larvae are gregarious: eating, molting and dispersing together.
5th - 7th ('middle')	Medium body size (4.87 to 7.93 mm). Previously molted head capsules present on the head of larvae. Fifth, 6th and 7th instar larvae have one, two and three head capsules present, respectively.
>7th ('old')	Large body size (> 9.94 mm). Larvae have more than 3 previously molted head capsules present. Males have a maximum of five head capsules, and females have seven head capsules.

Insect Culturing

I established a stock laboratory population of *U. lugens* from approximately 200 individuals (collected as egg clusters or first to second instar larvae) collected from *Eucalyptus* trees in Royal Park, Melbourne, Victoria. The stock population was cultured in an incubator under reverse-phase photoperiod 15L:9D light conditions at 25°C and approximately 70% humidity. The caterpillars were maintained with designed density (see below) in plastic containers (1 L) and fed with fresh, mature leaves of *Eucalyptus* spp., which were replaced every two days.

Manipulation of juvenile population density

First-instar larvae from the stock population were allocated to one of three experimental treatments that manipulated juvenile population density. The larvae were reared from the first instar until pupation in identical plastic containers (1L) at one of three densities: 'Low' density (one larva/container); 'Medium' density (five larvae/container); or 'High' density (25 larvae/container). Containers were supplied with fresh, mature leaves of *Eucalyptus spp.*, which were replaced every two days. Individuals were provided with *ad libitum* food in each treatment. Upon pupation, individuals were sexed by size (male pupae are smaller than females) and then transferred to individual vials (40 x 60 mm, 120 ml) and maintained under

the same environmental conditions as larvae until adult emergence. Individuals derived from the different density treatments were haphazardly allocated to one of two experiments examining either female calling behaviour or female pheromonal attractiveness. Only one experimental female from each medium- or high-density container was used for each experiment, in order to avoid pseudo-replication.

Juvenile density-dependent calling strategies of virgin females

I examined whether females derived from different juvenile rearing densities adjust their investment in sex pheromone signalling by varying both the time of onset and duration of calling behaviour. Female *U. lugens* typically eclose several hours before scotophase, and then commence calling shortly after their wings have expanded and continue calling for several hours into the scotophase. Calling is unambiguous in *U. lugens* as the female adopts a posture that exposes her pheromone gland. Newly-emerged virgin females from the different density treatments, Low (n= 24), Medium (n = 21) and High (n = 28) were placed individually in clear plastic containers (40 x 60 mm, 120 ml) and were recorded for their calling behaviour for the first 9 hours of the scotophase over four consecutive days. Females were observed on the hour, every hour over the 9-hour trial period and I noted whether or not they exhibited the calling posture. Observations were conducted under a red-filtered light to mimic the scotophase. I recorded, for each female, the time of each calling event, the number of calling bouts (consecutive one-hour blocks of calling), and the total number of one-hour blocks in which females were calling.

The effect of female juvenile population density on pheromonal attractiveness

I assessed whether juvenile rearing environment affects female pheromone investment by examining male preferences for pheromones from females reared at two different population densities: Low (L) from individuals reared as solitary larvae and High (H) from individuals reared at 25 larvae/container. Male preferences were assessed using a glass y-tube olfactometer (Fig. 1).

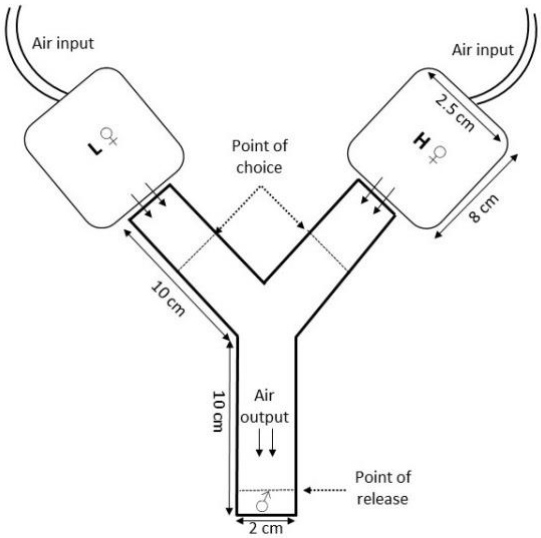

Figure 1. Schematic of the y-maze apparatus used to assess male preferences for sex pheromones from females reared at Low (L) or High (H) juvenile population density.

Here, a standardized, continuous air flow was introduced at the end of each arm of the y-maze, passing over the females and on to the receiving, focal male. A single, L female was placed in one arm and a single H female was placed in the other arm and left to acclimate for one hour. Both females were ≤ 48hrs post eclosion and were matched for body weight (mean ± standard error body weight difference = 3.84 ± 0.54 mg). After both females commenced calling, a virgin male from the stock population (≤ 36 hrs post eclosion, reared at a Medium population density of five larvae/container) was introduced into the central arm of the y-maze, and was deemed to have responded when he either walked or flew toward the airflow, travelling at least 5 cm into one of the arms of the y-maze and remaining there for more than 1 min. If males did not make any movement after 30 mins or move toward the y-maze junction within 10 seconds following introduction, they were removed, discarded and replaced with another male (n = 4,

9 per cent of trials were excluded). Males were given 60 min to make a choice, and males that did not make a choice during this period were excluded from the analysis (n = 8, 18.2 per cent of trials were excluded). A male moth was used once only, and pairs of females were used for two trials only (with females never occupying the same arm of the olfactometer twice). The olfactometer apparatus was washed with soap and water and dried after each trial. The position of the low- and high-density females was rotated after each trial to remove positional effects. The trials were conducted during the middle of the scotophase, when moths are most active (personal observation).

Juvenile density-dependent morphology and longevity

To assess the impact of juvenile rearing density on body size and longevity, a single male and single female was taken from each experimental container on the day of eclosion. Adults were sexed (females have filiform antennae, males have bipectinate antennae) and housed individually (container size: 40 x 60 mm, 120 ml). Adults were observed daily until their death, and were then preserved in ethanol (70%). Wing length was used as an index of body size (Miller, 1977). The left front wing was cut at its joint with the thorax. Scales were removed by dipping each wing in household bleach for 40-60s, and then by rinsing them in distilled water. Each wing was laid flat between micro-slides and then scanned at the resolution of 2400 dpi resolution (Epson Perfection V800 scanner). The length of the second-most posterior vein was measured from its point of insertion with the thorax to its termination at the distal edge of the forewing, using imageJ software (McNamara et al., 2008).

Statistical Analysis

All statistical analyses were conducted in R studio, version 3.5.2. For all models containing interaction terms, non-significant interactions were removed from final models (Engqvist, 2005). For all General Linear (Mixed) models, I optimally power transformed all dependent variables to maximize normality of model residuals, and the exponents used were noted with every analysis. Post-hoc comparisons were performed with Tukey's HSD.

The effect of age class on the juvenile population in the field was explored using a Generalized Linear Mixed Model (GLMM - package 'lme4') (Bates et al., 2015), with a Poisson distribution, and sampling date incorporated as a random effect.

The impact of population density on body size and longevity was explored using a General Linear Model.

The effect of juvenile population density on the likelihood that a female called was explored using a Generalized Linear Mixed Model, with a binomial distribution, while the effect of juvenile population density on the time of onset of calling in each scotophase was analysed with a GLMM, with a Poisson distribution. Both mixed models incorporated female identity as a random effect and used female relative wing size as a covariate. Here, I used standardized wing size calculated separately for each population density treatment (individual wing length—mean population wing length/ standard deviation of population wing length).

The total number of hours spent calling by a female across each of the four scotophases was explored using a General Linear Mixed Model, with a normal distribution. Not all females were eclosed for the entire first scotophase, and so I expressed calling duration as a proportion of the time that females were eclosed and able to call each day, hereafter 'proportion of time calling'. In this model, female relative wing size was used as a covariate and female identity was incorporated as a random effect.

Male olfactory preference trials were analysed using chi-square tests. For every analysis, I examined if males exhibited a directional bias (left or right arm of the olfactometer). These were uniformly non-significant and are not reported.

RESULTS

Demography of U. lugens *in natural populations*

U. lugens in Melbourne are bivoltine, with the first generation of first instar larvae emerging in January, and adults eclosing in May and the second larval cycle commencing shortly thereafter. Caterpillars of *U. lugens* are highly gregarious in the first to fourth instars ('Young' age), then gradually disperse from the fifth to seventh instars ('Middle' age), and switch to a solitary phase in the final nine or tenth (for male) or eleventh (for female) instars ('Old' age) (Fig. 2).

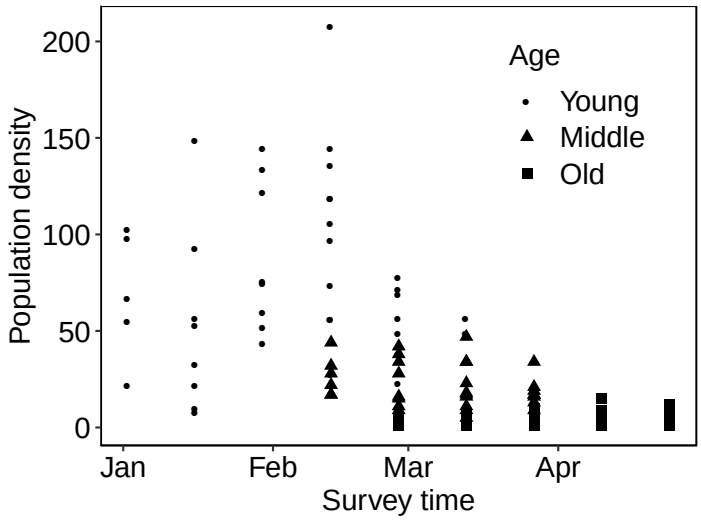

Figure 2. Distribution of *U. lugens* population density (number of individuals per group) classed by caterpillar's age: Young (1-4th instar larvae) (n = 41); Middle (5-7th instar larvae) (n = 32) and Old (> 7th instar larvae) (n = 50).

The distribution of population density was affected by the age class of the group (χ^2 = 828.89, n = 123; p < 0.001; Fig. 3). Post-hoc tests reveal that population density consistently decreased with increasing larval age (Fig. 3).

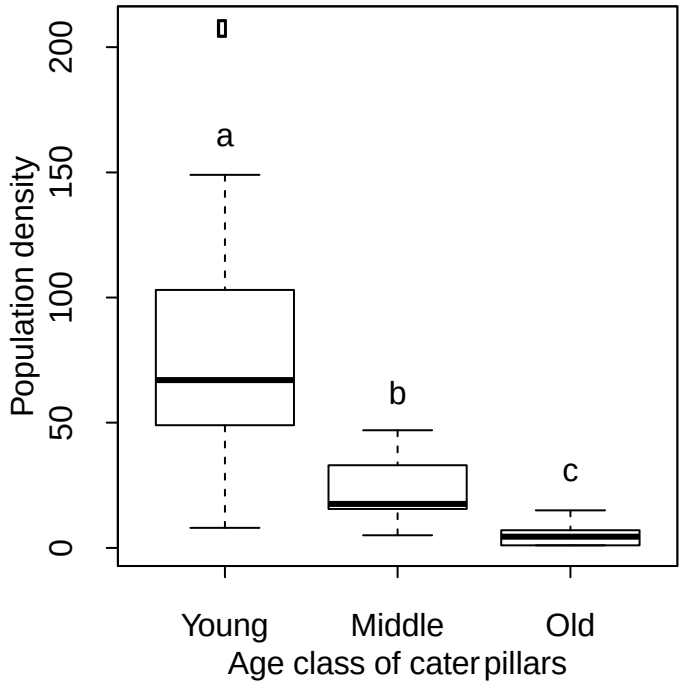

Figure 3. Median and interquartile range of population density (number of individuals per group) of larvae from Young (1-4th instar larvae) (n = 41), Middle (5-7th instar larvae) (n = 32) and Old (> 7th instar larvae) (n = 50) age classes. Different letters denoted significantly different comparisons.

The effect of juvenile density on adult morphology and longevity

Adult longevity (raised to the exponent 0.88) was affected by sex ($F_{1,186}$ = 47.87, p < 0.001; Table II), with males living longer than females. Longevity, however, was not affected by population density ($F_{2,186}$ = 0.89, p = 0.41; Table II). A non-significant interaction between sex and population density was removed from the final model ($F_{2,184}$ = 0.33, p = 0.72). Adult body size (raised to the exponent 1.32) was greater for females ($F_{1,186}$ = 854.21, p < 0.001; Table II) and was affected by population density ($F_{2,186}$ = 10.78, p < 0.001). Post-hoc tests revealed that individuals from high-density populations were larger than both medium- and low-density populations, but medium- and low-density populations were not significantly different from

each other (Table II). A non-significant interaction between sex and population density was removed from the final model ($F_{2,184} = 0.21$, $p = 0.81$).

Table II. Mean ± standard error of male and female body size and longevity for Low, Medium and High juvenile population density treatments. Sample sizes are displayed in parentheses.

Population density	Body size ± SE (mm)		Longevity (days)	
	Females	Males	Females	Males
Low	10.30 ± 0.16 (30)	7.59 ± 0.11 (33)	8.00 ± 0.31 (30)	10.24 ± 0.42 (33)
Medium	10.54 ± 0.11 (31)	7.82 ± 0.10 (31)	7.55 ± 0.46 (31)	9.77 ± 0.46 (31)
High	10.87 ± 0.11 (34)	8.08 ± 0.09 (31)	7.77 ± 0.35 (34)	10.65 ± 0.56 (31)

Juvenile density-dependent calling strategies of virgin females

The likelihood of a female calling on any given day was not affected by juvenile population density ($\chi^2 = 0.26$, $p = 0.88$). The likelihood of a female calling decreased with female age ($\chi^2 = 7.28$, $p = 0.06$). A non-significant interaction between juvenile population size and female age was removed from the final model ($\chi^2 = 0.82$, $df = 6$, $p = 0.99$). The likelihood of calling was not affected by relative female size ($\chi^2 = 0.06$, $df = 1$, $p = 0.80$).

The latency until calling within each scotophase (raised to the exponent 0.09) was significantly affected by an interaction between juvenile population density and female age (χ^2 = 12.59, df = 6, p = 0.05; Fig. 4). Post-hoc tests revealed that on the first day (one-day old), females eclosed from high juvenile population densities started calling earlier than the females eclosed from low juvenile population densities (Fig. 4). Furthermore, the latency until calling was reduced for relatively larger females (χ^2 = 7.35, df = 1, p = 0.007).

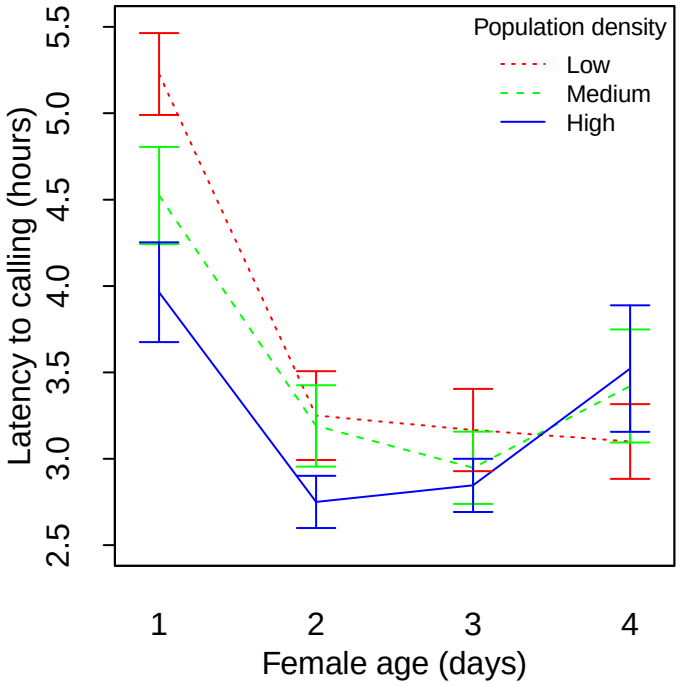

Figure 4. The effect of the interaction between population density (Low, Medium and High) and female age (one to four-day old) on the latency to female calling.

The proportion of the scotophase spent calling (raised to the exponent 1.72) was significantly affected by an interaction between population density and female age (χ^2 = 18.83, df = 6, p = 0.004). Post-hoc tests reveal that on the first day, females from high-density populations called for a longer duration than females from low-density populations (Fig. 5). Furthermore, the proportion of the scotophase spent calling was greater for relatively larger females (χ^2 = 4.92, df = 1, p = 0.03).

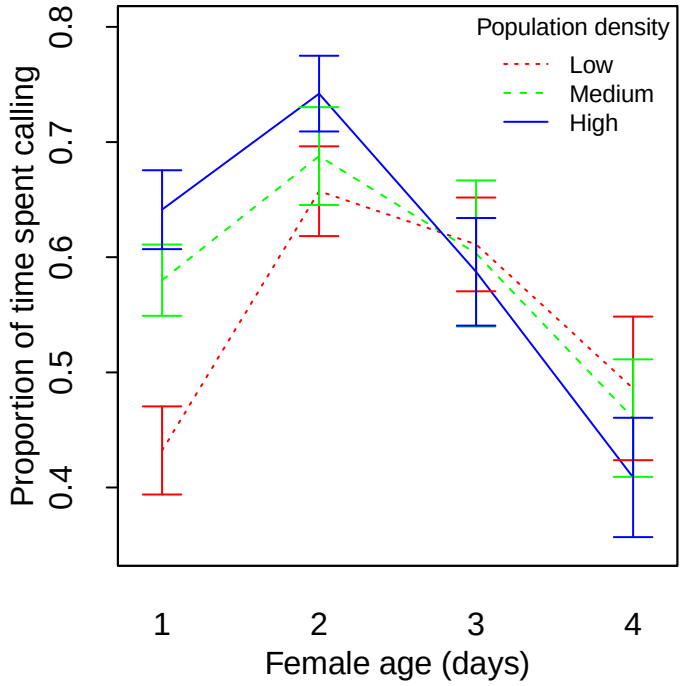

Figure 5. The effect of the interaction between population density (Low, Medium and High) and female age (one to four-day old) on the proportion of the scotophase spent calling.

Male preferences for pheromones from young or old females

Thirty-two males (72.7 per cent) made a successful choice between females from Low and High population densities. Males showed a significant preference for the pheromones produced by females derived from high-density populations (χ^2 = 4.50, p = 0.03; Fig. 6).

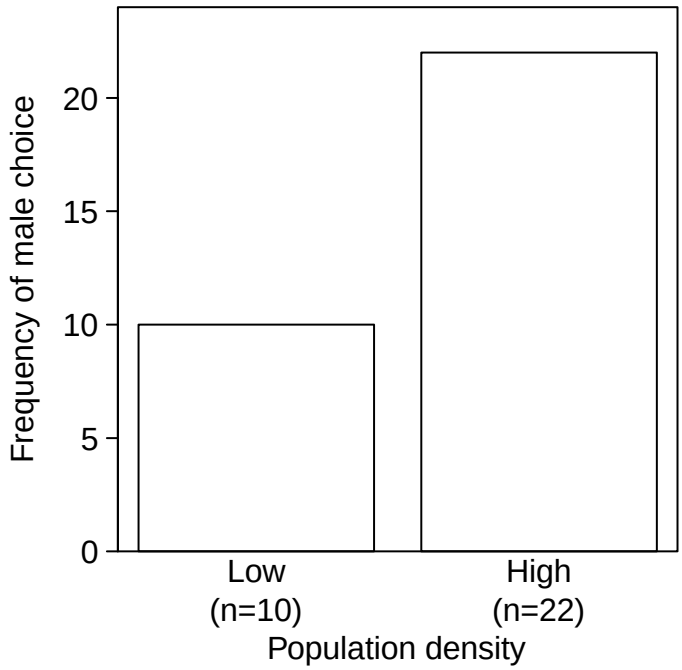

Figure 6. Frequency of male choice for females reared at either Low or High population densities and matched for body weight (n = 32 trials).

DISCUSSION

Females may adjust their investment in sexual signalling with changes in population density in response to the risk of reproductive failure, which may manifest through Allee effects or through competition with other females. My experiments reveal that the latter is more important in *U. lugens*. Females that eclosed from high juvenile population densities commenced calling earlier in the first scotophase and typically called for a longer time. Furthermore, males showed a significant preference for the pheromones of females that had eclosed from high than low juvenile population densities, suggesting that females alter not only the timing of pheromone release, but also the nature of their sex pheromones. Given that individuals were isolated at pupation, females were not exposed to conspecific calling during the experiments. Thus, these data reveal that females adjust their investment into mate

attraction through olfactory channels in response to *perceived* future competition with other females, which is informed by juvenile population density.

Competition between females may increase the risk of reproductive failure (Umbers et al., 2015), and altering the onset of calling and/or increasing calling duration in the scotophase may reduce this risk of competition from other signallers (Palaniswamy & Seabrook, 1985). This risk may be especially relevant for species such as *U. lugens*, where females are relatively immobile and there are temporal constraints on mating (Harris, 1975). Female *U. lugens* that eclosed from high population densities commenced calling behaviour several hours earlier in the evening compared with females from low population densities, and thus also called for a greater proportion of the scotophase. This response extends the "mating window" in which they may encounter males (see Fromhage et al. (2016), who were most active in the early scotophase (H.T.P; personal observation). More males are likely to be searching early in the scotophase because copulation can take up to four hours (H.T.P; personal observation) and males can mate only once per day. Males of *U. lugens* are likely to be polygynous as they live as adults from 8-13 days, and so males that mate early in the scotophase have more time to replenish their sperm supply before seeking females the next day. Interestingly, this pattern changed for older females, with the calling duration of females raised in high population density treatments being shorter than those from low population density treatments. These data, together with the result that females commenced calling earlier and spent more time on calling during the first scotophase, are consistent with a fitness cost of releasing pheromone (Harari et al., 2011; Jaffe et al., 2007).

Other lines of evidence indicate that female moths adjust their signalling behaviour in response to the potential for competition (Harari et al., 2011; Palaniswamy & Seabrook, 1985; Rehermann et al., 2016; Stelinski et al., 2006). For example, females of the noctuid moth *Pseudaletia adultera* grouped with conspecifics were more likely to call, commenced calling earlier in the scotophase, and spent more time calling than solitary females (Rehermann et al., 2016). Similarly, the oriental fruit moth *Grapholita molesta* called several hours earlier in the scotophase in the presences of a calling neighbouring females (Stelinski et al., 2006), and the proportion of female Eastern spruce budworm, *Choristoneura fumiferana*, that called was significantly higher when exposed to conspecific sex pheromones (Palaniswamy & Seabrook, 1985). The armyworm moth *S. littoralis* also increase the calling duration and calling more in later scotophase to attract more mates (Sadek et al., 2012). These data are consistent with the

view that increased investment in calling with higher densities of signallers is advantageous (Evenden et al., 2015; Gascoigne et al., 2009; Lim & Greenfield, 2007; Lim et al., 2007; Palaniswamy & Seabrook, 1985; Regniere et al., 2013). This most likely arises because less intensive signalling guarantees the female will not attract a male, rather than because individuals that signal more intensively are more likely to attract a male.

The preferences of male *U. lugens* for pheromones produced by females eclosed from high population densities provides compelling evidence that females are responding to perceived future competition with other females. The mechanistic basis for this male preference is unclear, but presumably arises from differences in pheromone quantity or structure (component ratio). Chemical analysis of the pheromone output of females reared under different juvenile population density treatments is beyond the scope of this study, but field experiments indicate that the concentration of pheromones may be important: males attracted to traps with a solitary female *U. lugens* had longer antennae than those attracted to traps with two females (Johnson et al., 2017b), suggesting that females from the low population density released lower concentrations of pheromone than those reared under high population density. Theory predicts that under conditions of male competition, females benefit from releasing small quantities of pheromone as this may attract higher quality males (Elgar et al., 2019), but it may pay females to release a greater quantity of pheromone when there is competition for males.

It is surprising that female investment in signalling behaviour was not greater for females raised in a low-density environment, given that the mating success of female moths is typically lower at lower population densities. For example, the rate of female mating success decreased with decreasing population density in spruce budworms, *Choristoneura fumiferana* (Regniere et al., 2013) and forest tent caterpillar moths, *Malacosoma disstria* (Contarini et al., 2009; Evenden et al., 2015). Nevertheless, while producing more, or more concentrated pheromones may travel further or persist for longer (Kokko & Rankin, 2006), evidence for a greater investment in pheromones with decreasing population density is equivocal. The concentration of (E)-11-hexadecenal (a sex pheromone component) emitted by the pickleworm, *Diaphania nitidalis,* was more concentrated at low than high densities of adult females (Valles et al., 1992). In contrast, female armyworm moths, *Spodoptera littoralis* (Sadek et al., 2012) and *Spodoptera exigua* (Yang et al., 2009) delay calling in the presence of conspecific pheromones, and the percentage of calling females of two leafrollers moths,

Choristoneura rosaceana and *Argyrotaenia velutinana* was reduced by half when exposed to conspecific pheromones (Gokce et al., 2007). The likelihood of calling does not change in the presence of conspecific competitors in other species (El-Sayed & Suckling, 2005; Stelinski et al., 2006). Experimental evolution studies revealed that juvenile population density did not create a selection pressure on the calling likelihood of the Indian meal moth, *Plodia interpunctella* (Ashman et al., 2016). These results suggest that female responses to social cues are highly species-specific and may differentially affect various components of female calling behaviour.

In many insect species, larvae developing at high densities often take longer to pupate, and the adults are smaller in size with reduced fecundity and longevity (Bhavanam & Trewick, 2017; Gage, 1995; Johnson et al., 2017a; McNamara et al., 2010). For example, male Mediterranean flour moths, *Ephestia kuehniella* reared at high juvenile population density, were smaller, produced fewer eupyrene (fertilising) sperm, but mated more frequently than males reared at low densities (Bhavanam & Trewick, 2017). Similarly, male and female almond moths *Cadra cautella* reared at high density took longer to develop and were smaller than those reared at low density (McNamara et al., 2010). However, in this study, adult male and female *U. lugens* were larger at higher population densities, in contrast to previous studies of *U. lugens* (Johnson et al., 2017a) and other species (Bhavanam & Trewick, 2017; McNamara et al., 2010). Juvenile population density may also have a negligible effect on adult body size, as in the Indian meal moth, *Plodia interpunctella* (Gage, 1995). Comparisons across species and experimental designs should be treated with caution, because the relationship between population density and adult morphology may be non-linear: the adult body size of *U. lugens* at very high population density (twice that of this study) decreased (Johnson et al., 2017a). Such non-linear relationships between larval density and body size have been noted in other moth species (Ronnas et al., 2010). There are several reasons why larger adults may emerge from more dense larval populations. First, the larvae of *U. lugens* may have greater feeding efficiency when living in groups (Fiorentino et al., 2014; Fitzgerald & Peterson, 1988; Pescador-Rubio, 2009), which may also explain why the species is gregarious. Second, selection may favour larger adult body size at high densities: increased wing size may improve the dispersal capacity of males, allowing them to avoid competition by seeking mating opportunities elsewhere. Larger body size in females may provide a competitive edge, because the pheromones of a larger females are preferred by males in other lepidopteran species (Rhainds

et al., 1995; van Dongen et al., 1998). Importantly, however, I controlled for female body size in the assays of female pheromonal attractiveness, thus, the preference by males for females derived from high-density populations is not due to their larger size, per se.

In conclusion, my experiments show, for the first time, that female investment into chemical signalling behaviour responds to social cues in their juvenile environment that indicate levels of competition for mates. When reared at high density, females were larger, increased their calling behaviour and produced pheromones that were more attractive to males. Given that female longevity is short and thus the risk of reproductive failure is high, there is a premium on being able to attract a mate quickly when the competition for mates is high. This strategic investment in calling behaviour in *U. lugens* challenges the widespread assumption that the cost of chemical signalling is negligible.

CHAPTER 4

Diet-dependent reproductive investment in gumleaf skeletonizer moths, *Uraba lugens*

ABSTRACT

Juvenile diet can profoundly affect subsequent adult development, morphology, and reproductive investment. Yet, little is known about how juvenile diet affects adult investment into chemical-based sexual signalling, perhaps due to the historical assumption that pheromone production is not costly. I explored how juvenile diet influenced reproductive investment of both female (the quality of female sex pheromone) and male (pre- and post-copulatory) adults in the gumleaf skeletonizer, *Uraba lugens* a significant moth pest. Juvenile diet was manipulated by either rearing individuals on different species of Eucalypt host plants (*Eucalyptus camaldulensis* and *Eucalyptus moorei*) that differed in macro-nutrient content. Additionally, juveniles were reared on *E. moorei* with two fertiliser treatments (fertilised as a rich nutritional condition and non-fertilised as a poor nutritional condition). First, chemical foliar analysis showed that the leaf carbon content in *E. moorei* was higher than that in *E. camaldulensis*, but there was no inter-specific difference in nitrogen content. While the fertiliser treatment resulted in an increased leaf nitrogen content, there was no difference in carbon content. Second, I found that the effect of juvenile diet on adult fitness depended upon adult sex: in females, diet influenced body size, while in males diet influenced longevity. Juvenile diet also affected female pheromonal attractiveness: y-maze olfactometer assays showed that males tended to prefer the pheromones produced by females reared on fertiliser-fed host plants. Finally, host plant species affected male pre-copulatory investment: males reared at *E. camaldulensis* have longer antennae but less dense sensilla, although there was no difference in the testes size of males reared on the two different species. There was also no difference in the reproductive traits of males reared on plants with different fertiliser treatments. Taken together, the results of my experiments are consistent with the view that juvenile diet affects reproductive signalling investment differently for males and females. Adult females allocate nutrients to body size, which reflects fecundity and pheromone quality for mate attractiveness. In contrast, males allocate nutrients to longevity and antennae size, both of which improve mate search and mating success.

INTRODUCTION

Generally, nutrition is advantageously allocated to a variety of different functions in animals, and diet-dependent reproduction investment typically differs between the sexes. Females may invest in larger, more nutrient-rich or more eggs (Awmack & Leather, 2002; Bonoan et al., 2015; Carrière, 1992; Moreau et al., 2006) whereas males may invest in larger testes or spermatophores (Delisle & Hardy, 1997; Dewsbury, 1982; McNamara et al., 2009; Svard & Wiklund, 1989; Wedell et al., 2002). There is increasing interest in how diet affects investment in chemical communication (Henneken et al., 2017; Henneken & Jones, 2017; Henneken et al., 2015), which has important implications for insect sex pheromones (Reddy & Guerrero, 2004). In both capital breeding species, where eggs are formed prior to eclosion, and income breeding species, where adults forage, juvenile nutrition plays a vital factor in determining adult fitness (Boggs, 2009; Carsten-Conner et al., 2010; Darragh et al., 2019). For example, the pheromone composition of the butterfly *Heliconius melpomene* is affected by larval host plants but not adult diet (Darragh et al., 2019). The effects of juvenile diet on sex pheromones have been reported in diverse insects including beetles (Rantala et al., 2003a; Xue et al., 2016), cockroaches (Clark et al., 1997) and lepidoptera (Conner et al., 1990; Darragh et al., 2019). Hence, pheromones can be considered as condition-dependent signals (Rantala et al., 2003a), and the intended receivers should alter their mate choice responses accordingly (Maynard Smith & Harper, 2003).

Host plants may be critical in determining the nature of sex pheromones in several ways (Reddy & Guerrero, 2004). First, they can influence the synthesis of sex pheromones (Conner et al., 1990; Hendry, 1976; Landolt & Phillips, 1997; McNeil & Delisle, 1989). For example, oak leaf roller moths, *Archips semiferanus,* reared on artificial diet do not produce an important pheromone component, (Z)-10-tetradecenyl acetate, which is produced by females reared on oak leaves (Hendry et al., 1975), while the quantities of different components of the pheromone of medflies, *Ceratitis capitata,* are modified by diet (Merli et al., 2018). Second, the presence of suitable host plants plays a role in stimulating the release of sex pheromones in some (Hendrikse & Vosbunnemeyer, 1987; Riddifor & Williams, 1967), but not all moths (Gomez & Rojas, 2006). While several studies have explored the impact of host plant quality on adult fitness (Moreau et al., 2017), few have investigated whether variation in host-plant nutrients can influence investment in key adult life-history traits. Micro- and macro-nutrients

vary considerably between different host plants, and the species, age of plant, and soil nutrient profile can influence the level of nutrient intake in phytophagous insects (Mattson, 1980).

Nitrogen is widely implicated as a critical, and frequently limiting, nutritional element for the development of many herbivorous insects (Mattson, 1980), evidenced by numerous experimental studies using fertiliser supplements (Chen et al., 2008; Slansky & Feeny, 1977; Zhu et al., 2019) to manipulate the levels (both quantity and quality) of soluble nitrogen compounds, see Mattson (1980). The effect of nitrogen on growth is particularly important for herbivorous insects, such as lepidopterans, that are often nitrogen-limited because of the low nitrogen content of their host plants (Slansky & Feeny, 1977). Nutrient limitation is predicted to impact the reproductive traits of both sexes, particularly when males provide nutrient-rich spermatophores (Simmons & South, 2012). For example, a juvenile diet high in nitrogen increases lifetime fecundity of female *Ostrinia nubilalis* (Bonoan et al., 2015), while nitrogen levels while males that received elevated nitrogen levels developed larger in walnut flies *Rhagoletis juglandis* (Carsten-Conner et al., 2010). In lepidopterans, male fitness may depend on dietary nitrogen that determines ejaculate traits (Macartney et al., 2019). Nevertheless, the high nitrogen level in diet can also create negative impacts in insects such as lepidoptera (Kurze et al., 2018) and grasshopper (Zhu et al., 2019), due to an imbalance between their intake target and the nutrient content of their food (Behmer, 2009; Simpson & Raubenheimer, 2012).

Diet can also influence investment in the sensory structures of adult insects, including the number and type of chemoreceptors (Bernays & Chapman, 1998; R. F. Chapman & Lee, 1991; Elgar et al., 2018; Safonkin et al., 2004) which are crucial for detecting chemical signals and cues (R. F. Chapman, 1982). For example, grasshoppers, *Schistocerca americana,* fed lettuce have more sensilla on their antennae than those fed an artificial diet (R. F. Chapman, 2002). Surprisingly, few studies have investigated the effects of diet on antennae size or chemoreceptor density in lepidoptera, despite the extensive investigations of pheromones in this taxon (Symonds & Elgar, 2008). Nevertheless, the impact of other environmental factors, such as juvenile density, on investment in male antennae have been documented for insects (Greenwood & Chapman, 1984; Johnson et al., 2017a).

The gumleaf skeletonizer moth, *Uraba lugens* is a capital breeding species that does not feed as an adult, and so the reproductive life-history parameters are determined by the resources obtained during the juvenile period. *U. lugens* is a phytophagous moth that feeds on over 103 myrtaceous trees in which the genus *Eucalyptus* is dominant (Berndt & Allen, 2010).

Early studies indicate that the suitability of eucalypt host plants for larval development (mortality and development time) is correlated with measures associated with adult performance, including pupal weight and fecundity (Campbell, 1962). More recent studies reveal that juvenile density influences investment in adult reproductive communication, include pheromone attractiveness in females (chapter 3), and in antennae and testes size in males (Johnson et al., 2017a, 2017b).

Here, I explore the consequences of different diets on the reproductive traits of both males and females of *U. lugens*. Specifically, I compare these traits in adults that had been reared on either of two *Eucalypt* host plant species that apparently differed in nutritional quality (Bell & William, 1997), and of adults reared on one of these host plant species that had or had not been treated with a commercial fertiliser. First, I analysed the concentration of nitrogen and carbon in foliar to confirm the two species differed in nutrient availability. Second, I examined the effects of juvenile diets on adult body size and adult longevity. Third, I examined the functional impact of different juvenile diets on female pheromonal attractiveness by testing male preferences of the odours of females reared on different diets. Finally, I measured pre-copulatory (antennae length and sensilla density) and post-copulatory (testes size) reproductive traits of males reared on different diets. I predicted that diets with a greater concentration of nitrogen and carbon will enhance (a) female reproductive output and therefore her attractiveness to males, and (b) male pre and post-copulatory reproductive traits.

MATERIAL AND METHODS

Insect culturing

Egg clusters of the Australian gumleaf skeletonizer *U. lugens* were collected from several locations in Melbourne, Victoria, and transferred to the laboratory where they were maintained in plastic containers (1L, one egg cluster per container) in an incubator (15L:9D light conditions at 22.5°C and approximately 70% humidity) until they hatched. The larvae were then housed in plastic containers (1L), supplied with fresh, mature leaves of *Eucalyptus* spp. that were replaced every two days, until they reached third instar.

Host plants stock

Two species of host plants, *E. camaldulensis* and *E. moorei*, obtained from Kuranga Native Nursery, Victoria, were maintained in 110L plastic pots (Reko) in the glass house (School of BioSciences, The University of Melbourne, Parkville, Victoria, Australia), and watered once or twice per week and fertilised once per 6 months with a commercial slow-release fertiliser (Osmocote). In total, 15 plants of *E. camaldulensis* and 47 plants of *E. moorei* were used for culturing the insect stocks. All of the plants used in the experiments were less than two years old.

Host plant species effects

The second and third instar larvae from the same egg cluster were allocated evenly between the potted plants of *E. moorei* (n = 15 plants) and *E. camaldulensis* (n = 15 plants), which were watered every week. Larvae that reached the 7th instar (identified by the presence of three head-capsules) were then transferred to small plastic containers (1L, 10 individuals per container) and maintained in the incubator as above. The larvae were fed with the leaves of their treatment host plant species until pupation. The pupae were transferred to individual vials (40 x 60 mm, 120 ml), where the moths eclosed.

Fertiliser treatment effects

Second and third instar larvae were raised on potted plants of *E. moorei* that had been allocated to one of two treatments: non-fertilised (water only, n = 17 plants) or fertilised fortnightly (commercial Aquasol soluble fertilizer with 9g in 4.5 litres of water, n = 15 plants). Larvae that reached the 7th instar were transferred to small plastic containers (1L, 10 individuals per container), which were kept in incubator conditions as above. The larvae were fed with the leaves of their treatment host plant until pupation. Pupae were then transferred to individual vials (40 x 60 mm, 120 ml), where the moths eclosed.

Analysis of foliar nutrients in juvenile diets

The percentage of nitrogen and carbon in the leaves was examined at the Farquhar Isotope Laboratory, Research School of Biology, Australian National University. The leaf samples were collected three times during the experimental period (beginning, middle and end of the experiment) with 10 leaves randomly sampled from across the plants (n = 30 for each diet

treatment). The samples were dried at 70° C for 72 hours, weighed and ground for further analysis. The C and N concentrations in the leaves were measured in 5mg dried samples. Leaf C: N concentration was measured by a standard protocol with a continuous-flow isotope ratio mass spectrometer–Micromass Isochrome CF-IRMS (Micromass, Middlewich, England).

Tin-wrapped samples were loaded into carousels that were placed on an AS200 autosampler (Carlo Erba, Milan) that drops a sample in response to a pulse of compressed air. The sample falls into the laboratory-built chromium oxide-packed column in an approximately 100 ml min^{-1} flow of high purity helium with a pulse of oxygen and combusts at 1050˚C. Cobaltous silver oxide at the base of the column removes halides. The oxidised gases were then swept through a column packed with copper wire, held at 650˚C where excesses oxygen was removed and oxygen was stripped from oxidised nitrogen species. Water was then scrubbed out in a magnesium perchlorate scrubber before the gases were separated in a Porapak QS 50-80 mesh (Alltech, Baulkham Hills, NSW, Australia) ¼" packed stainless-steel column fabricated by the laboratory, and held at 25°C. Nitrogen gas exits from the Gas chromatography, followed by carbon dioxide after about one minute, as measured by a Thermal Conductivity Detector (TCD). The gases flow to an open split where they were sucked into the mass spectrometer through a 75 µ silica capillary.

The mass spectrometer (MS) was controlled by Micromass proprietary software (version EA v1.64). MS data were used for assessing the amount of nitrogen in the sample and TCD data were used for carbon analysis, because the MS is more sensitive for the small sample of nitrogen whereas carbon dioxide is diluted for measurement by the MS and is therefore less reliably measured after the TCD. The response of the TCD and MS were determined by running a range of standard materials, of known composition. From these, graphs were constructed of peak area *versus* amount of nitrogen (percentage in material * mass) and linear regressions with a slope and offset calculated. From these, the nitrogen content and blank for each sample were calculated ((Peak_Height * Slope) ± Offset)/Mass.

All samples were run against laboratory standard materials, which were combusted and processed in the same way to ensure the samples were calibrated and weighed to produce peaks of a similar amplitude. For carbon a C_3 Beet (δ = -24.62 ‰ VPDB) and a C_4 cane (δ = -10.45 ‰ VPDB) sucrose were used. Beet is a well-calibrated in-house standard (analysed many times against IAEA CH-3 cellulose paper) and ANU sucrose is identical to IAEA CH-6. For nitrogen, several standards including a gelatine, glycine, Cysteine and pure alanine (supplied

by the University of Oxford), as well as a calibrated fishing nylon (also used as a carbon standard) were used. To extend the carbon and nitrogen scales, USGS Glutamic Acids 40 and 41 were used. The data were corrected using the slope and offset calculated from the standard materials.

Juvenile diet and adult longevity and morphology

To assess the impact of juvenile diet on body size and longevity, one male and female were taken from each experimental container on the day of adult eclosion. Adults were sexed (females have filiform antennae, males have bipectinate antennae), housed individually in a vial (40 x 60 mm, 120 ml) and observed daily until their deaths. Adults do not feed or drink. The deceased individuals were preserved in ethanol (70%). Wing length was used as an index of body size. Here, the left forewing of each male was cut as close as possible to the base and dipped in light bleach liquid for 30s (in order to remove the scales). Wings were laid flat between two coverslips and digital images were obtained at 2400 dpi resolution using an Epson Perfection V800 scanner. The length of the second-most posterior vein was measured from its point of insertion with the thorax to its termination at the distal edge of the forewing, using ImageJ software (Schneider et al., 2012).

Juvenile diet and adult female attractiveness

I conducted male preference tests, using a y-tube olfactometer (Fig. 1), to assess whether juvenile diet affects the attractiveness of adult female pheromones. I compared male responses to the pheromones of females reared on the different diets for each experiment: different host plant species (*E. camaldulensis and E. moorei*), and different fertilised treated host plant (non-fertilised and fertilised). The pairs of females were ≤ 48hrs post eclosion and were matched for body weight (mean +/- standard error body weight difference = 4.34 +/- 0.67 mg in different host plant species and 5.35 +/- 0.43 mg in different fertiliser treatments).

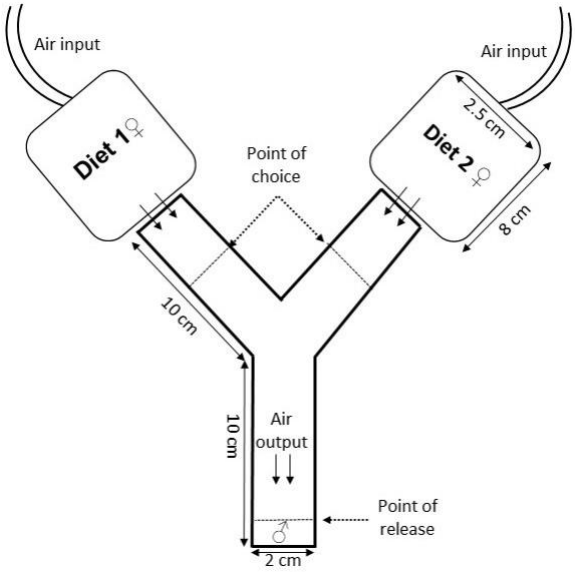

Figure 1. Schematic of the y-maze apparatus used to assess male preferences for sex pheromones from females that were reared at different diets.

A standardized, continuous air flow was introduced at the end of each arm of the y-maze, passing through the containers with the calling females and on to the receiving male. A single female raised on one diet was placed in one container and a female from the complementary diet was placed in the other container, and both were left to acclimate for one hour. Once both females had commenced calling, a virgin, stock male (≤ 36 hrs post eclosion, reared at an intermediate population density of five to ten larvae/container with various eucalyptus leaves collected from parks) was introduced at the base of the y-maze, and was deemed to have responded when he either walked or flew toward the airflow, travelling at least 5 cm into one of the arms of the y-maze and remaining there for more than 1 min. Males that did not make

any movement after 30 mins or moved to one of the arms in less than 10 seconds after being introduced were replaced with another male and excluded from further analysis. The trial lasted 60 min. Each male was used once only, and pairs of females were used for two trials only (with females never occupying the same arm of the olfactometer twice). The olfactometer apparatus was washed with soap and water and dried after each trial. The position of the low- and high-density females was rotated each trial to remove positional effects. The trials were conducted during the middle of the scotophase, when moths are most active (personal observation).

Juvenile diet and male reproductive investment

To assess the effect of juvenile diet on male pre- and post-copulatory reproductive investment, I killed adult males two-days post-eclosion (when the testes are fully-developed) and measured both antennal morphology and testes size. Males were stored in 70 per cent ethanol until required.

The left antenna of each male (n = 15 raised on *E. camaldulensis* and n = 16 raised on *E. moorei*) was removed using microscissors and gently placed on a two-sided carbon sticky tab (12.0 mm diameter, Proscitech) and mounted on an electron microscopy stub (12.6 mm diameter, Proscitech). The antennae were imaged with low-vacuum uncoated scanning electron microscopy (SEM) on a FEI Quanta 200F scanning electron microscope at the Bio21 Advanced Microscopy Facility (Bio21 Institute, The University of Melbourne, Parkville, Victoria, Australia). For all specimens, a spot size of 2.0 and pressure of 0.80 mbar were used, with a high voltage of 10.0 kV. These SEM images were used to measure sensilla density, the length (from tip to scape) and the total number of flagellomeres. These measurements were obtained using imageJ software (Schneider et al., 2012).

Following Hansson (1995), six types of sensilla were detected on the antennae of the males, based on their external morphology, and included trichodea, basiconica, coeloconica, auriculate, chaetica, and styloconica (Fig. 2). Sensilla trichodea are the dominant type of sensilla in all of the flagellomeres of the male antenna and was the only type found in the antennal branches, suggesting that these sensilla are significant. Sensilla trichodea have numerous pores and it is widely thought that their main sensory channel is olfactory, and these sensilla have been implicated in mate search in tortricid moths (Triseleva & Safonkin, 2006). Sensilla basiconica, coeloconica and auriculate are involved in host plant odour perception

(Denotter et al., 1978; Triseleva & Safonkin, 2006). Sensilla chaetica and styloconica do not have pores on the surface and are therefore unlikely to function as olfactory receptors. Accordingly, I measured the density of trichodea only, and for convenience refer to this as sensilla density.

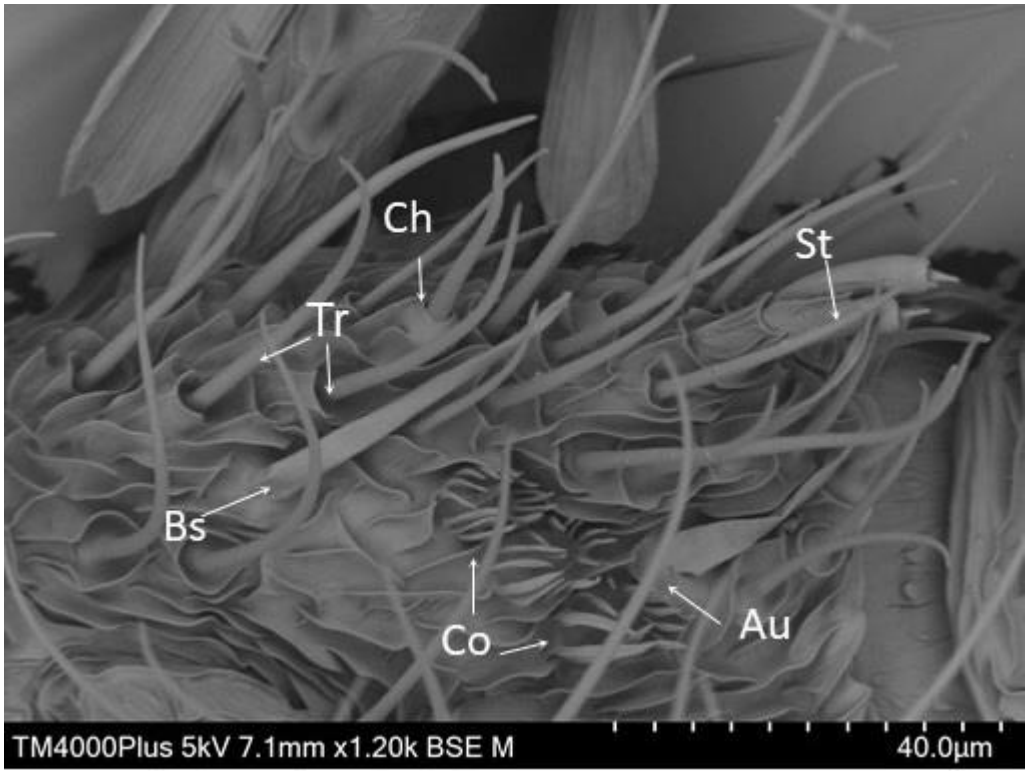

Figure 2. Different types of sensilla located on the ventral side of one segment: the long sensilla, trichodea (Tr), were the dominant types of sensilla; sensilla chaetica (Ch) are similar to Tr but the basal socket is different; sensilla auricillica (Au) have a flat shape and are wide at the base; Sensilla coeloconia (Co) have a grooved peg surrounded by 12-16 spines; and sensilla styloconica (St) are peg-like, set on top of a conical cuticular style found at the distal margin of each flagellomere.

The average sensilla density for each male, based on the density of sensilla trichodea of three flagellomeres (1st, 15th and 35th from the antennal tip) was calculated by dividing the total number of sensilla trichodea observed in those three flagellomeres by the total area in µm^2 of those flagellomeres.

Each male was dissected to obtain a measure of testes size. The testes is a fused spherical organ located in the 6-9th abdominal segment (Johnson et al., 2017a). The testes were removed with microscissors and placed on a slide. The testes were then imaged immediately (to avoid desiccation) with a Sony camera (ILCE-QX1) under an Olympus microscope (SZX16) at x 5

magnification. The images were then measured using ImageJ (Schneider et al., 2012). The repeatability of the measurements was high: the corelation coeficient for testes size measurement was 99.38% (n = 30). Wing length was used as an index of body size and measured as above.

Statistical Analysis

All statistical analyses were conducted in R studio, version 3.5.2 (R_Core_Team, 2018). I used general linear models to examine the impact of host plant species and supplementary fertilizer on the percentage of nitrogen and carbon in the foliar, with the mass of each leaf sample incorporated as a covariate.

I explored the impact of juvenile diet on male and female body size and longevity using general linear models, with a diet x sex interaction term.

Male olfactory preference trials were analysed using chi-square tests. For each experiment, I initially examined if males exhibited a directional bias (left or right arm of the olfactometer), but these were uniformly non-significant and are not reported.

I investigated the impact of juvenile diet on the reproductive investment of adult males (antennae length, number of flagellomeres, density of sensilla and testes size) with general linear models that included wing size and a diet x relative wing size interaction term.

For all models containing interaction terms, non-significant interactions were removed from the final models (Engqvist, 2005). For all general linear models, I optimally power transformed all dependent variables to maximize normality of model residuals, and the exponents used were noted with every analysis. For all figures, untransformed means ± standard errors, and median ± interquartile ranges are presented throughout, for ease of comparison.

Variation in nitrogen and carbon composition of leaves

The effect of Eucalypt species host plant

The concentration of nitrogen in the leaf samples (raised to the exponent 0.04) did not differ between the species of eucalypt (mean ± standard error for concentration of nitrogen in *E. moorei* = 1.59 ± 0.14; *E. camaldulensis* = 1.43 ± 0.12; $F_{1,27} = 0.84$, $\beta = 0.004 \pm 0.005$, $p = 0.37$), and was not affected by the mass of the leaf sample ($F_{1,27} = 0.01$, $\beta = -0.005 \pm 0.05$, $p = 0.91$).

The concentration of carbon in the leaf samples (raised to the exponent 2.64) was significantly higher in the leaves of *E. moorei* than those of *E. camaldulensis* ($F_{1,27} = 19.39$, $\beta = 4635.00 \pm 1053.00$, $p < 0.001$; Fig. 3), but not affected by the mass of the leaf sample ($F_{1,27} = 0.83$, $\beta = -8936.00 \pm 9802.00$, $p = 0.37$).

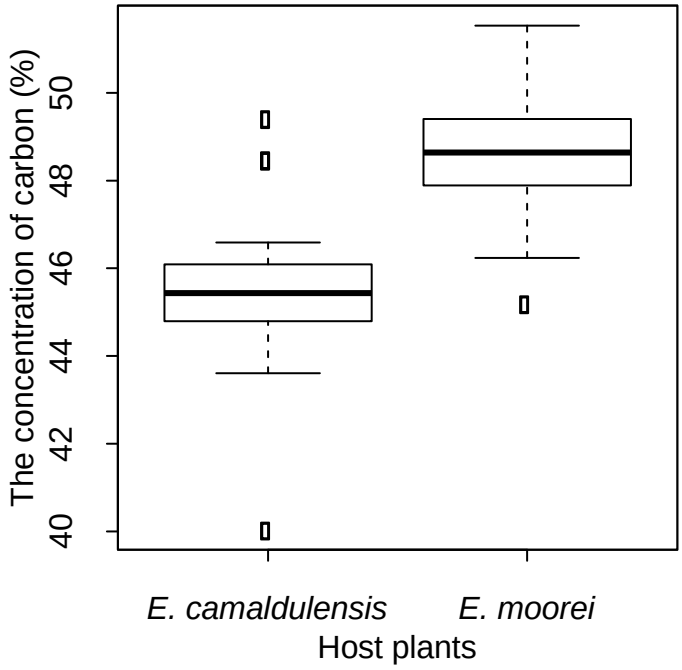

Figure 3. Median and interquartile range of the total carbon per mg leaves of *E. camaldulensis* and *E. moorei* (n = 30 each).

The effect of fertilizer treatment

The concentration of nitrogen present in the leaf sample (raised to the exponent 0.04) was significantly lower in fertilized than in non-fertilized leaves ($F_{1,26}$ = 5.24, β = -0.007 ± 0.003, p = 0.03, Fig. 4), and was not affected by the mass of the leaf sample ($F_{1,26}$ = 0.32, β = -0.02 ± 0.03, p = 0.57).

The concentration of carbon (raised to the exponent 0.04) was not affected by the fertilizer treatment (mean ± standard error for concentration of carbon in fertilised plant = 47.02 ± 0.29; non-fertilised plant = 46.94 ± 0.25; $F_{1,27}$ = 0.22, β = -0.001 ± 0.0004 p = 0.64), or by the mass of the leaf sample ($F_{1,27}$ = 1.74, β = -0.005 ± 0.004, p = 0.20).

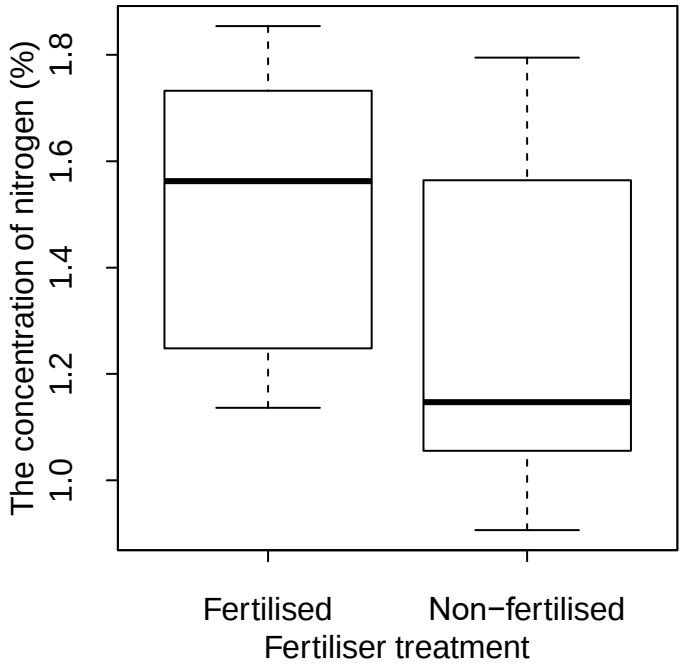

Figure 4. Median and interquartile range of the total nitrogen content per mg (dry mass) in leaves of fertilised and non-fertilised *E. moorei* (n = 30 each).

The effect of juvenile diet on adult morphology and longevity

The effect of Eucalypt species host plant

The variation in adult body size (raised to the exponent 2.08) was explained by the host plant species x adult sex interaction term ($F_{1,117}$ = 11.27, p = 0.001). Post-hoc tests revealed that females were larger than males, and individuals raised on *E. moorei* were significantly larger

than those raised on *E. camaldulensis*. The interaction term reflects the greater effect of diet on body size for females than males.

The variation in adult longevity (raised to the exponent 1.2) was explained by the host plant species x adult sex interaction term ($F_{1,117}$ = 8.12, p = 0.005; Fig. 5). Post-hoc tests revealed that host plant species did not affect female longevity, but that males lived longer than females and that males raised on *E. camaldulensis* lived longer than males raised on *E. moorei* (Fig. 5).

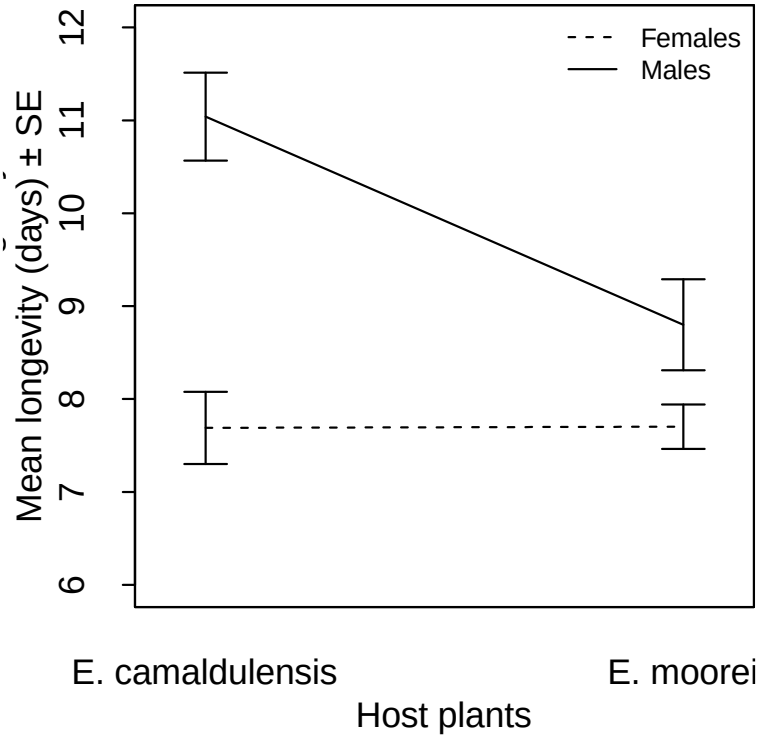

Figure 5. Mean longevity (days) ± SE of male and female reared on *E. camaldulensis* and *E. moorei* host plants

The effect of fertilizer treatment

The variation in adult body size (raised to the exponent 1.12) was explained by a significant fertiliser treatment x adult sex interaction term ($F_{1,131}$ = 7.69, p = 0.006; Fig. 6). Post-hoc tests revealed that females fed on fertiliser-treated plants were significantly larger than females fed on non-fertiliser-treated females, but this was not the case for males (Fig. 6).

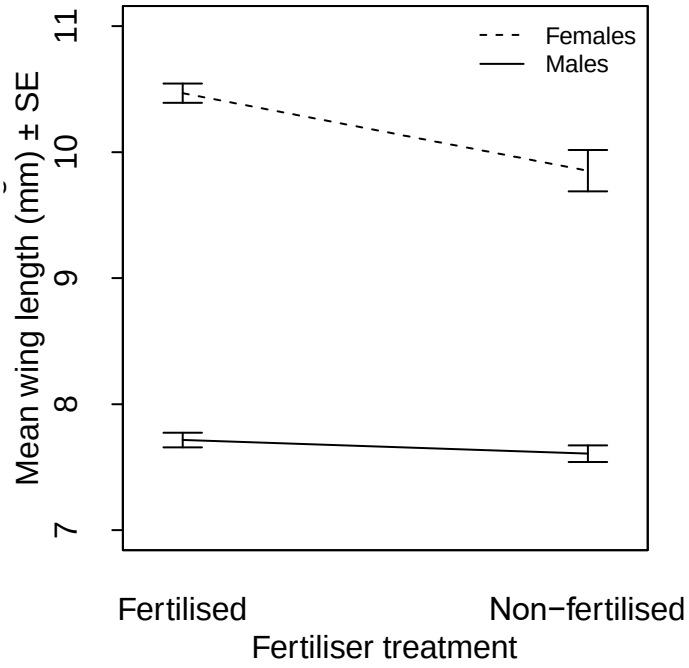

Figure 6. Mean wing length (mm) ± SE of males and females reared on fertilised and non-fertilised treated *E. moorei*.

Adult longevity (raised to the exponent 0.64) was affected by fertiliser treatment ($F_{1,130}$ = 6.99, β = -0.34 ± 0.13, p = 0.009) and sex ($F_{1,130}$ = 18.60, β = 0.54 ± 0.12, p < 0.001). Post-hoc tests revealed that individuals fed on fertiliser-treated plants lived significantly longer than individuals fed on non-fertiliser-treated plants, and that males lived longer than females. A non-significant interaction between fertiliser treatment and sex was removed from the final model ($F_{1,129}$ = 0.33, p < 0.57).

Juvenile diet and female attractiveness

The y-maze olfactometer choice tests revealed that when males were given a choice between the odour from females (mean ± standard error body weight difference = 4.34 ± 0.67 mg) reared on a diet of *E. camaldulensis* or *E. moorei* leaves, twenty-six males (from 30 trials) made a successful choice, but there was no consistent preference for the pheromones produced by females raised on either diet ($\chi^2 = 0.15$, $p = 0.69$; Fig. 7).

When males were given a choice between females (mean ± standard error body weight difference = 5.35 ± 0.43 mg) fed a diet of fertilised or non-fertilised plants, twenty-seven males (from 32 trials) made a successful choice. There was a trend for males to prefer the pheromone produced by females that had been raised on fertilizer-treated plants ($\chi^2 = 3.0$, $p = 0.08$, $n = 27$; Fig. 7).

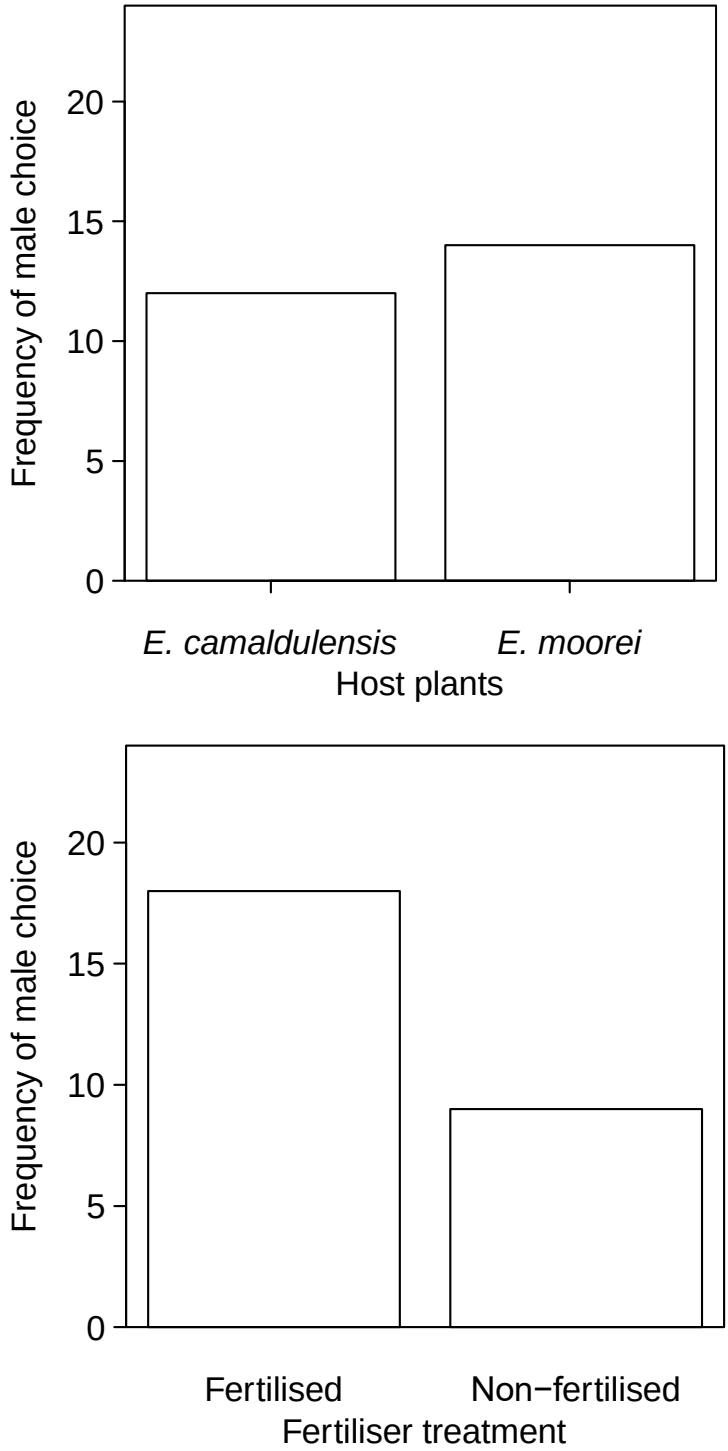

Figure 7. Frequency of male choice for females reared on different juvenile diets: Eucalypt plant species (upper) and fertiliser treatment (lower).

Juvenile diet and male reproductive investment

The effect of Eucalypt species host plant

The length of antennae (raised to the exponent 0.04) of males raised on *E. moorei* was longer than that of males raised on *E. camaldulensis* ($F_{1,27}$ = 16.21, β = 0.003 ± 0.001, p = 0.0004; Fig. 8). Interestingly, the length of antennae was not affected by wing size ($F_{1,27}$ = 2.75, β = 0.0006 ± 0.0004, p = 0.11). A non-significant host plant species x wing size interaction term was removed from the final model ($F_{1,26}$ = 0.16, p = 0.69).

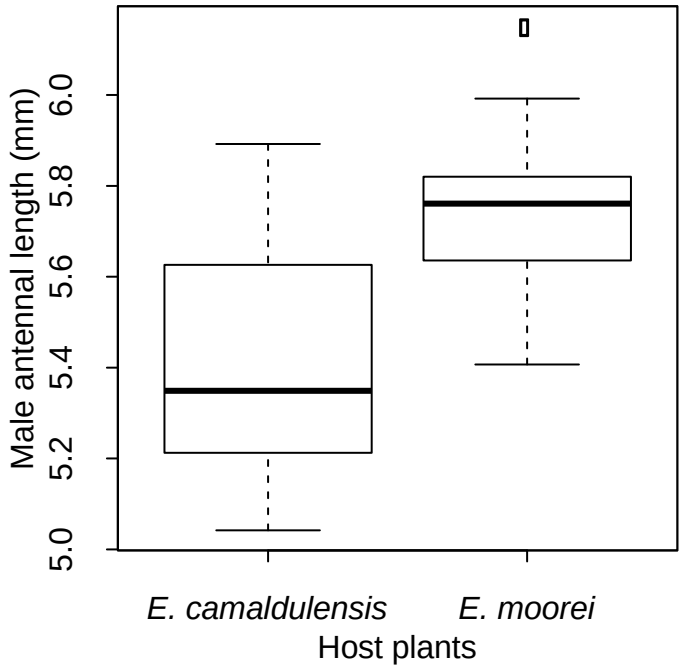

Figure 8. Median and interquartile range of male antennal length when fed on different host plant species: *E. camaldulensis* (n = 15) and *E. moorei* (n = 16).

The number of flagellomeres (raised to the exponent 3.48) was not affected by either host plant diet (mean ± standard error number of flagellomeres: *E. moorei* = 48.75 ± 0.57; *E. camaldulensis* = 49.00 ± 0.59; $F_{1,27}$ = 0.04, β = -9104 ± 44518, p = 0.84) or wing size ($F_{1,27}$ = 0.51, β = 18322 ± 25683, p = 0.48). A non-significant host plant species x wing size interaction term was removed from the final model ($F_{1,26}$ = 0.28, p = 0.60).

The density of antennal sensilla (raised to the exponent 1.16) was greater for males raised on *E. camaldulensis* than for males raised on *E. moorei* ($F_{1,27}$ = 6.02, β = -2.17e-04 ± 8.86e-05, p = 0.02, Fig. 9), but was not affected by wing size ($F_{1,27}$ = 0.05, β = -1.11e-05 ± 5.11e-05, p = 0.83). A non-significant host plant species x wing size interaction term was removed from the final model ($F_{1,26}$ = 0.07, p = 0.80).

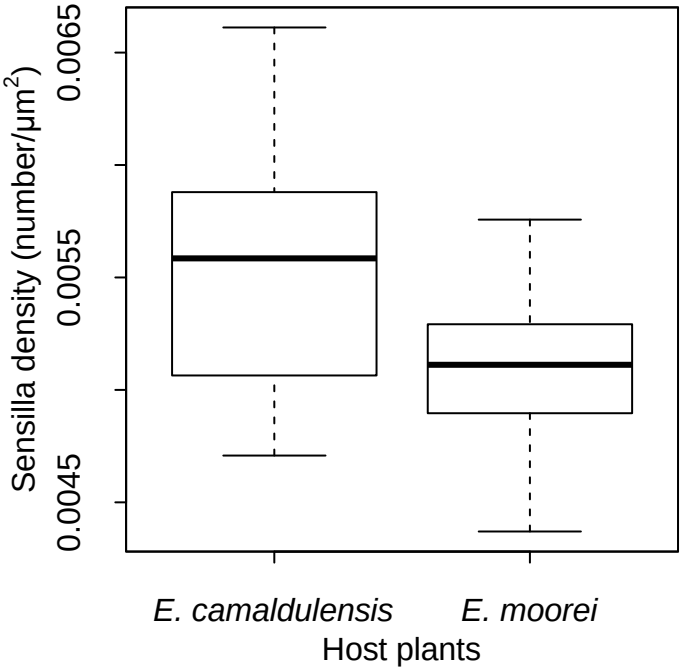

Figure 9. Median and interquartile range of male sensilla density when were fed on different host plant species: *E. camaldulensis* (n = 15) and *E. moorei* (n = 16).

The variation in testes size (raised to the exponent 0.08) was not explained by the host plant diet (mean ± standard error testes size (mm^2): *E. moorei* = 0.25 ± 0.01; *E. camaldulensis* = 0.23 ± 0.01; $F_{1,27}$ = 2.75, β = 0.01 ± 0.006, p = 0.11) nor by male wing size ($F_{1,28}$ = 3.49, p = 0.07). A non-significant host plant species x wing size interaction term was removed from the final model ($F_{1,26}$ = 2.89, p = 0.10).

The effect of fertiliser treatment

The length of antennae (raised to the exponent 0.84) was not affected by the host plant fertiliser treatment (mean ± standard error antennal length (mm): fertilised = 5.70 ± 0.09; non-fertilised = 5.73 ± 0.07; $F_{1,28}$ = 0.085, β = 0.02 ± 0.06, p = 0.77), but was positively correlated with wing size ($F_{1,28}$ = 12.67, β = 0.11 ± 0.03, p = 0.001). A non-significant fertiliser status by wing size interaction term was removed from the final model ($F_{1,27}$ = 0.19, p = 0.66).

Similarly, the number of flagellomeres (raised to the exponent 0.2) was not affected by the host plant fertiliser treatment (mean ± standard error number of flagellomeres: fertilised = 43.22 ± 0.60; non-fertilised = 44.15 ± 0.53; $F_{1,28}$ = 1.25, β = 0.009 ± 0.008, p = 0.27) or wing size ($F_{1,28}$ = 0.004, β = -0.0003 ± 0.004, p = 0.95). A non-significant fertiliser status by wing size interaction term was removed from the final model ($F_{1,27}$ = 0.38, p = 0.54).

Finally, testes size (raised to the exponent 0.84) was not affected by the host plant fertiliser treatment (mean ± standard error testes size (mm^2): fertilised = 0.273 ± 0.016; non-fertilised = 0.270 ± 0.019; $F_{1,28}$ = 0.01, β = -0.003 ± 0.03, p = 0.92) or wing size ($F_{1,28}$ = 0.01, β = -0.002 ± 0.01, p = 0.91). A non-significant fertiliser status by wing size interaction term was removed from the final model ($F_{1,27}$ = 0.02, p = 0.90).

DISCUSSION

Adult life-history and reproductive traits in the capital breeding, gumleaf skeletonizer moth, *U. lugens,* are affected by juvenile diet. My experiments reveal how larval diet differences in either carbon (i.e., comparisons between host plant species) or nitrogen (fertilised or non-fertilised host plants) content have different consequences for adult males and females. In general, diet influenced female fecundity through differences in adult body size, while diet influenced male longevity and their capacity to find reproductive partners. Adult females were larger if they had been reared on diets with greater nitrogen and carbon content, but while the former females produced sex pheromones that tended to be more attractive to males, differences in carbon content did not influence pheromone attractiveness. Adult male traits were influenced by the level of carbon, but not nitrogen content, in the larval diet: greater levels of carbon content resulted in shorter lifespan, but longer antennal length. While juvenile diet can have profound effects on male testes size in insects (Svard & Wiklund, 1989), and in some cases creates a trade-off between pre-and post-copulatory traits (Durrant et al., 2016;

Simmons & Emlen, 2006), the testes size of male *U. lugens* did not change with increasing amounts of carbon or protein in the juvenile diet.

The influence of macro-nutrients on the adult reproductive traits of female *U. lugens* are broadly consistent with nutrient limitation theory (Mattson, 1980), as the level of both carbon and nitrogen content in the juvenile diet influences female body size and thus fecundity (Awmack & Leather, 2002; Bonoan et al., 2015; Garcia-Barros, 2000). For example, female tortricid moths, *Choristoneura rosaceana*, reared on a nutritious diet have a larger body size and hence lay more eggs (Carrière, 1992), while female larvae of the Melissa blue butterfly, *Lycaeides melissa,* that fed on a poor-quality host plant eclosed as smaller adults and were neglected by males (Forister & Scholl, 2012). In contrast, these macro-nutrients did not have a consistent influence on adult male traits: antennal size and sensilla density changed with carbon content only, and longevity decreased with higher carbon content. Nevertheless, both these macro-nutrients had no influence on post-copulatory investment. This result is not consistent with previous empirical studies indicating that males often allocate more resources towards testes or ejaculates. For example, males of the European grapevine moth, *Lobesia botrana* fed on a high-quality host plant were larger and transferred larger spermatophores (Muller et al., 2015). Similarly, walnut flies *Rhagoletis juglandis* reared on a rich nutrient diet produced larger testes and larger ejaculates (Carsten-Conner et al., 2010). Interestingly, the absence of a treatment effect on male testes size in this study is consistent with my finding that male resource stressors, via an immune challenge, also do not impact male testes investment (Chapter 5).

There is increasing evidence that sex pheromones are affected both qualitatively and quantitatively by larval diet, and thus can act as 'honest' signals to attract mates (Henneken et al., 2017; Henneken & Jones, 2017; Henneken et al., 2015). My behavioural assays showed that given a choice, males tended to prefer the odours derived from females raised on a diet with a higher nitrogen content: the nitrogen level might reflect alkaloid production in the host plant (Landolt & Phillips, 1997), which may act as chemical precursors of the sex pheromones. This male preference is consistent with theory proposing that pheromones provide receivers with information about the quality of the signaller (Henneken et al., 2017). Additionally, a male may benefit from mating with females reared on a high-quality, nitrogen-rich diet because they may produce more, and better nourished eggs (Xie et al., 2015).

Interestingly, males showed no preference between females raised on different *Eucalyptus* host plant species, despite the inter-specific differences in carbon content. There are several explanations. First, the sex pheromones emitted by females reared on different *Eucalyptus* host plant species may not differ, despite the difference in carbon content affecting female body size. Second, the leaves of both *E. camaldulensis* and *E. moorei* may have sufficient carbon content that ensures the larvae can sequester the specific precursors to synthesise a similarly attractive pheromone. Previous studies indicate that both species of *Eucalyptus* are high-quality host plants for *U. lugens*, with relatively low mortality rates, short development times and heaviest pupae (Cobbinah, 1983, 1985; Cobbinah et al., 1982). Finally, the sex pheromones produced by females reared on these different host plant species may differ, but there may be no clear benefits to males that prefer females from one species over the other: larvae reared on *E. moorei* develop into larger adults with a shorter lifespan, while larvae reared on *E. camaldulensis* develop into smaller adults with a longer lifespan. It is possible that males can distinguish between the pheromones produced by females reared on different host plants, but their preferences for one over the other are context dependent.

Juvenile diet affects male longevity, and thus time spent mate-searching, but not consistently across different macro-nutrients. The positive correlation between nitrogen content and longevity is broadly consistent with nutrient limitation theory (Mattson, 1980), although it is not consistent with studies of other insects that report negative effects of nitrogen-rich diets (Kurze et al., 2018; Zhu et al., 2019). The geometric framework for nutrition (Simpson & Raubenheimer, 2012) may provide explanations for the negative correlation between adult male longevity and carbon content in the juvenile diet. This framework assumes that animals require a particular mixture of nutrients during the course of their life, and an imbalance between an individual's intake target and the nutrient content of its food may not necessarily result in improved performance (Behmer, 2009; Raubenheimer & Simpson, 1993): in this case, the diet incorporated a higher carbon content without a compensating higher nitrogen content. Further, high nutrient content diets may reduce insect performance by affecting specific physiological processes, such as increasing the metabolic costs of storing and excreting excess nutrients (Anderson et al., 2005; Boersma & Elser, 2006). Thus, "digestive efficiency" may decline with increasing nutrient content, and insects with a nutritionally poor diet will activate increasing "digestive efficiency" (Raubenheimer & Simpson, 1993).

Antennae size and the number of sensilla are important in chemical communication and likely respond to selection (R. F. Chapman, 1982; Elgar et al., 2018). Conventional wisdom holds that female moths release minute quantities of sex-pheromone (Symonds & Elgar, 2008; Wyatt, 2003), and so sexual selection will favour males with highly efficient chemical receptor organs, such as longer or more complex antennae and more sensilla (Elgar et al., 2019). Field experiments revealed that male *U. lugens* with longer antennae were better able to locate solitary, younger females (Johnson et al., 2017b), who may have greater reproductive value (Foster & Howard, 1999; Xu & Wang, 2009). Laboratory experiments revealed that male Neriid flies, *Telostylinus angusticollis,* that have relatively longer antennae (similar body size) were more likely to win male-male competitive interactions and also achieved matings more quickly (Fricke et al., 2015). Studies of several insects have also demonstrated the effects of host plants on the number and density of antennal sensilla (Bernays & Chapman, 1998; R. F. Chapman, 2002; R. F. Chapman & Lee, 1991; Rogers & Simpson, 1997; Safonkin et al., 2004). Males of *U. lugens* that were reared on carbon-rich *E. moorei* had longer antennae but lower sensilla density than males reared on *E. camaldulensis*. This suggests that while a diet with a higher carbon content allows males to increase the size of their antennae, the costs of the underlying neural networks (Niven & Laughlin, 2008; Stockl et al., 2016) may prevent investment in larger numbers of sensilla. Whether antennal length, per se, or the number of sensilla are most important in determining male chemical detection capacity remains to be tested in this species.

Together, the results of my experiments demonstrated that juvenile diet affects reproductive signalling investment differently for males and females: females allocate nutrients to body size, which affects fecundity and pheromone quality for mate attractiveness, while males allocate nutrients to longevity and antennae size, both of which improve mate search and mating success.

CHAPTER 5

A trade-off between investment into immunity and male antennal morphology and female reproductive output in gumleaf skeletonizer moths, *Uraba lugens*

ABSTRACT

Trade-offs between immunity and reproductive investment in invertebrates have been well documented. Yet, little is known about how activation of the immune system affects investment into chemical sexual signalling. While there is limited evidence of trade-offs between male pheromone production and immunity, how immune activation affects signal receiver traits, antennae, has never been investigated. I used the gumleaf skeletonizer moth, *Uraba lugens*, to explore how upregulation of immunity affects male antennal functional morphology and female pheromone quality, and other life-history traits. Here, I injected final-instar larvae with a high or low dose of either an immune elicitor or a control solution, in order to reveal potential dose-dependency in any trade-offs. Male trade-offs were explored by measuring multiple antennal traits, using scanning electron microscopy. Female pheromone quality was assayed by testing male preferences for pheromones produced by immune-challenged and control females in a y-maze olfactometer. Additionally, testes size and female ovary mass were measured. Immune activation affected male, but not female signalling investment: immune challenged males had a lower density of sensilla on their antennae, but female pheromonal attractiveness was not affected by their immune status. Immune activation, however, did reduce female investment into ovary mass. Interestingly, males and females both increased their longevity following an immune challenge, suggesting a shift in resources to increase the opportunity for mating.

INTRODUCTION

Immune system maintenance and up-regulation is costly (Kraaijeveld & Godfray, 1997; Lochmiller & Deerenberg, 2000; Zuk & Stoehr, 2002). Thus, organisms must allocate their finite resources between immune defence and other traits, such as reproduction (English & Bonsall, 2019; Fedorka et al., 2004; Lochmiller & Deerenberg, 2000; McKean & Nunney, 2001; Schwenke et al., 2016; Zuk & Stoehr, 2002). Theoretical and empirical research has revealed phenotypic and evolutionary trade-offs between immune investment and a suite of pre-copulatory and post-copulatory sexual traits (for a review, see Lawniczak et al., 2007; Schwenke et al., 2016). Studies in a range of taxa have demonstrated the impact of immune activation on invertebrate sexual signalling, including acoustic (Ahtiainen et al., 2005; Jacot et al., 2004; Simmons et al., 2005; Tregenza et al., 2006) and visual signals (Martinez-Lendech et

al., 2018; Rantala et al., 2000; Wormington & Luttbeg, 2018). The impact on chemical signalling is less understood (but, see Barthel et al., 2015; Chemnitz et al., 2017; Rantala et al., 2003a; Rantala et al., 2003b; Sadd et al., 2006; Worden et al., 2000; Worden & Parker, 2005) despite it being the dominant means of sexual signalling in insects (Johansson & Jones, 2007).

Studies documenting trade-offs between immunity and chemical signalling have focused entirely on the signaller's perspective (Barthel et al., 2015; Chemnitz et al., 2017; Rantala et al., 2003a; Rantala et al., 2003b; Sadd et al., 2006; Worden et al., 2000; Worden & Parker, 2005). In contrast, trade-offs in chemical receiving structures (antennae) have not been examined, despite the costs of maintaining signal receiving structures (Niven & Laughlin, 2008; Stöckl et al., 2016) and their obvious role in reproduction. Antennae length and the number of sensilla (chemoreceptors) they bear are important determinants of chemical signalling success (R. F. Chapman, 1982; Elgar et al., 2018; Symonds et al., 2012). Generally, female moths release minute quantities of sex-pheromone (Symonds & Elgar, 2008; Wyatt, 2003), so selection may favour males with antennal morphology, including antennal length and sensilla numbers, that optimises odorant-receptor interactions (Elgar et al., 2019; Symonds et al., 2012; Wang et al., 2018). For example, male gumleaf skeletonizer moths, *U. lugens,* with long-antennae were better able to locate younger females (Johnson et al., 2017b) which have a greater residual reproductive value (Foster & Howard, 1999; Xu & Wang, 2009). In addition, male flies *Telostylinus angusticollis* that have longer antennae (controlling for body size) were more successful at acquiring mates (Fricke et al., 2015). Previous studies have demonstrated plasticity in the functional morphology of antennal traits in response to diet or nutrient limitation (Bernays & Chapman, 1998; R. F. Chapman & Lee, 1991; Safonkin et al., 2004), and to population demography (Johnson et al., 2017a). For example, male *U. lugens* increase their investment in antennal size when reared at low juvenile population density (Johnson et al., 2017a), presumably to increase their ability to detect sparsely-located mates. Since sensilla are costly and condition dependent (Elgar et al., 2019), the ability of males to invest in these traits may trade-off with immune investment, as has been demonstrated with a range of other pre-copulatory sexually selected traits (Pomfret & Knell, 2006; Rantala et al., 2000).

Female moths, like other insects, rely on sex pheromones to reveal their location to males (Svensson, 1996). Historically, the costs to females of producing these signals was considered trivial (Alberts, 1992; Cardé & Baker, 1984; Fromhage et al., 2016; Kokko & Wong, 2007), despite accumulating evidence that female pheromone quality and calling (pheromone-

releasing behaviours) are condition-dependent (Foster & Johnson, 2011; van Dongen et al., 1998) and exhibit significant plasticity (Harari et al. (2011); Chapter 2, 3 and 4). Female sex pheromones are used as signals to attract males, and thus can affect her mating success and, in some instances, the quality of the mate she attracts (Harari et al., 2011; Johansson & Jones, 2007; Svensson, 1996; Symonds et al., 2012). Pheromone quality is condition dependent (Henneken et al., 2017; Henneken & Jones, 2017; Henneken et al., 2015; Rantala et al., 2003a), and therefore a trade-off is expected when limited resources must be partitioned between sexual signalling and the immune system (Kotiaho, 2001b; Rantala et al., 2003a). For example, the attractiveness of the sex pheromone produced by male grain beetles, *Tenebrio molitor,* was reduced after an immune challenge (Worden et al., 2000; Worden & Parker, 2005). However, very few studies have investigated trade-offs between female sex pheromones and immunity, likely due to the historical assumption that such pheromones are not costly to produce (Alberts, 1992; Cardé & Baker, 1984; Fromhage et al., 2016; Kokko & Wong, 2007). An exception is the study by Barthel et al. (2015), who showed that the composition of sex pheromone produced by female tobacco budworm moths, *Heliothis virescens*, changed following injection with a pathogenic immune elicitor, with females becoming less attractive and experiencing reduced mating success.

Males and females have different life-history strategies that are expected to be reflected in sexual dimorphism in base-line immune activity – Bateman gradients of immunity (Rolff, 2002; Zuk & Stoehr, 2002). Here, it is proposed that females gain fitness by maximizing their longevity, and thus by having a greater immune investment, which in turn increases the time available for egg production and oviposition (Rolff, 2002). Conversely, males are predicted to maximise their fitness by increasing their mating frequency and should thus invest in increasing their mating rates and/or their gametic investment, instead of immunity (Rolff, 2002). This sexual dimorphism depends on the relative reproductive investment between the sexes and can be reversed in species in which males invest more than females into reproduction (Vincent & Gwynne, 2014). Although not a clearly sex role-reversed taxon, in many Lepidoptera, males contribute significant spermatophores at mating. Typically, copulations are lengthy, and males can only mate at most once per night. In such cases, male longevity may become increasingly correlated with the number of matings he can acquire (Barthel et al., 2015).

A variety of immune elicitors are used by researchers to explore the trade-offs between immunity and other traits, including live bacteria, wounding and non-pathogenic immune elicitors. Lipopolysaccharides (LPS), are a non-pathogenic microbial immune elicitor, derived from the surface of gram-negative bacteria, allowing quantification of the costs of immune responses, without the confounding effects of physiological sickness. LPS induces upregulation of a number of invertebrate immune effector systems, including the phenoloxidase cascade (PO), which is involved in wound repair and in the encapsulation of foreign bodies, and lytic (lysozyme-like) activity, which is the major antibacterial defense (Jacot et al., 2005; McNamara et al., 2013b; Simmons, 2012). Previous studies that stimulated the immune system with LPS reveal trade-offs between immunity investment and reproductive traits, including spermatophore size (Kerr et al., 2010), fecundity (Ahmed et al., 2002), mating frequency (McNamara et al., 2013b), nuptial gifts (Fedorka & Mousseau, 2007; Fedorka & Sevgili, 2014; Fedorka et al., 2004), and sperm quality (McNamara et al., 2013a; Simmons, 2012).

The gumleaf skeletonizer moth, *Uraba lugens,* (Lepidoptera: Nolidae) is an ideal model to examine the trade-offs between immune investment and female chemical signalling and male sensory structures. Females have poor flight capacity (Harris, 1975), so their mating success depends largely on the attractiveness of their sex pheromones. Given that female re-mating rates are low in this species (18.34%) (Chapter 2), and that males can only mate once per night (personal observation), male fitness may be primarily determined by their longevity and their ability to locate receptive females quickly, rather than by postcopulatory selection. Furthermore, I have already demonstrated that female pheromone investment is facultatively adjusted in response to a range of factors, including female age (Chapter 2), population demography (Chapter 3) and diet (Chapter 4). Similarly, male *U. lugens* adjust their investment into pre-copulatory (antennae) and post-copulatory (testes size) traits, according to various factors, including population demography and diet. For example, males reared at low population densities invest more in antennae, improving their mate detection (Johnson et al., 2017a, 2017b), while juvenile diet affected investment into the functional morphology of the antennae (Chapter 4).

Here, I explored the relationship between immune challenges at the juvenile stage to reproductive investment in both male and females. I injected final-instar caterpillars with different doses of a non-pathogenic immune elicitor or a control. First, I investigated the impact of those immune challenges on life-history traits, such as developmental rate, body size

and longevity. Second, I examined the impact of immune challenge on male pre- (antennal morphology) and post-copulatory (testes size) reproductive investment. Third, I explored the functional impact of this variation on male mate location behaviour, by testing male preferences for odours produced by females that had received different immune challenges at juvenile stage. I predicted that immune challenged individuals should demonstrate reduced investment into secondary sexual traits and reproductive output, in a dose-dependent manner.

MATERIALS AND METHODS

Insect culturing

Egg clusters of the Australian gumleaf skeletonizer moth *U. lugens* were collected from multiple locations in Melbourne, Victoria in May 2019 and transferred to the laboratory for hatching and culturing in an incubator (15L:9D light conditions at 22.5°C and approximately 70% humidity). The caterpillars were maintained in plastic containers (approximately 40-50 individuals per 1 L container) until the fifth instar (identified by the presence of a head-capsule), after which individuals were moved to a 1 L container at a density of 10 individuals /container. Containers were supplied with fresh, mature leaves of *Eucalyptus* spp., which were replaced every two days.

Immune assays

Recently-molted, final instar individuals (male: ninth instar; females: tenth instar) were haphazardly collected from the stock population and weighed prior to allocation to the immune-challenge treatments: a 'high' dose of a non-pathogenic immune elicitor, a lipopolysaccharide (LPS) derived from *Serratia marcescens* (Sigma-Aldrich L6136); a 'low' dose of LPS; or a control solution of an isotonic ringer (Grace's insect medium; Sigma- Aldrich G8142), to account for the potential immune response to the injection itself.

The LPS dose was derived from a review of the literature of LPS doses used to elicit an immune response in multiple invertebrate species, taking into account species-specific body masses (Adamo, 1999; Ahmed et al., 2002; Jacot et al., 2005; McNamara et al., 2013a; Moret & Schmid-Hempel, 2000; Schuhmann et al., 2003). I used sex-specific mean weights of a cohort of final-instar *U. lugens* to calculate the relative dose for this species (high dose = 0.08 µg of LPS/mg of larvae; low dose = 0.06 µg of LPS/mg). Thus, each male was injected with either 3.80

µg of LPS (high dose) or 2.66 µg of LPS (low dose) dissolved in 1.5 µl of ringer. For females, immune-challenged caterpillars were injected with either 5.99 µg of LPS in 1.5 µl of ringer (high dose) or 4.19 µg of LPS (low dose) dissolved in 1.5 µl of ringer. For both males and females, control treatment individuals were injected with 1.5 µl of ringer. Individuals were injected using a 2.5 µl micro-syringe (Hamilton 7632-01) with a 33-gauge needle (Hamilton 7803-05) in the rear proleg on the day they moulted into their 9^{th} (for males) or 10^{th} (for females) instar larvae. After injection, caterpillars were kept individually in 1L plastic container and fed with *Eucalyptus* spp. leaves (which were replaced every two days) until pupation. Individuals were monitored daily for mortality. Pupal weight was measured 7 days after pupation (in order to avoid damaging newly-pupated individuals) and were then transferred to individual vials (40 x 60 mm, 120 ml) and maintained under the same environmental conditions as larvae until adult emergence. After eclosion, adults were observed for longevity (adults do not feed or consume water). Individuals that failed to eclose were also recorded.

Effect of immune challenge to pre-adult mortality, pupal duration, longevity and body size.

Individuals were checked daily for pupation and adult eclosion, and any pre-adult (before pupation and before adult eclosion) mortality was recorded. After adult eclosion, adults were individually housed in 120ml containers until their death, and their longevity recorded. Individuals were preserved in ethanol (70%). Wing length was used as an index of body size. Here, the left forewing of each male was cut as close as possible to the base and dipped in light bleach liquid for 30 s (in order to remove the scales). Wings were laid flat between two coverslips and then scanned at the resolution of 2400 dpi resolution (Epson Perfection V800 scanner). The length of the second-most posterior vein was measured from its point of insertion with the thorax to its termination at the distal edge of the forewing, using imageJ software (Schneider et al., 2012).

Effect of immune challenge on male's pre- and post-copulatory reproductive investment

To assess the effect of immune challenge on male pre- and post-copulatory reproductive investment, emerged adult males were killed two-days post-emergence (when testes are fully developed) and their antennal morphology and testes size was measured. The left antenna of approximately 30 males from each experimental treatment were removed using microscissors and gently placed on a 12.0 mm double-sided carbon sticky tape (Proscitech IA023) and

mounted on a 12.6 mm electron microscopy stub (Proscitech G040). These antennae were imaged with low-vacuum uncoated scanning electron microscopy (SEM) on a FEI Quanta 200F scanning electron microscope at the Bio21 Advanced Microscopy Facility (Bio21 Institute, The University of Melbourne, Parkville, Victoria, Australia). For all specimens, a spot size of 2.0 and pressure of 0.80 mbar were used, with a voltage of 10.0 kV. These SEM images were used to measure sensilla density (number of sensilla per μm^2 antenna), the length (from tip to scape) and the total number of flagellomeres which were obtained using ImageJ software (Schneider et al., 2012).

Following Hansson (1995), I detected six types of sensilla, based on their external morphology, on the antennae of the males, and these included trichodea, basiconica, coeloconica, auriculate, chaetica, and styloconica (Fig. 1). Sensilla trichodea are the dominant type of sensilla in all of the flagellomeres of the male antenna and was the only type found in the antennal branches, indicating that these sensilla are important. Sensilla trichodea have numerous pores and so their main sensory channel is widely regarded as olfactory, and they have been implicated in mate search in tortricid moths (Triseleva & Safonkin, 2006). Sensilla basiconica, coeloconica and auriculate are involved in host plant odour perception (Denotter et al., 1978; Triseleva & Safonkin, 2006). Sensilla chaetica and styloconica do not have pores on the surface and are therefore unlikely to function as olfactory receptors. Accordingly, I measured the density of sensilla trichodea only and, for convenience, refer to this as sensilla density.

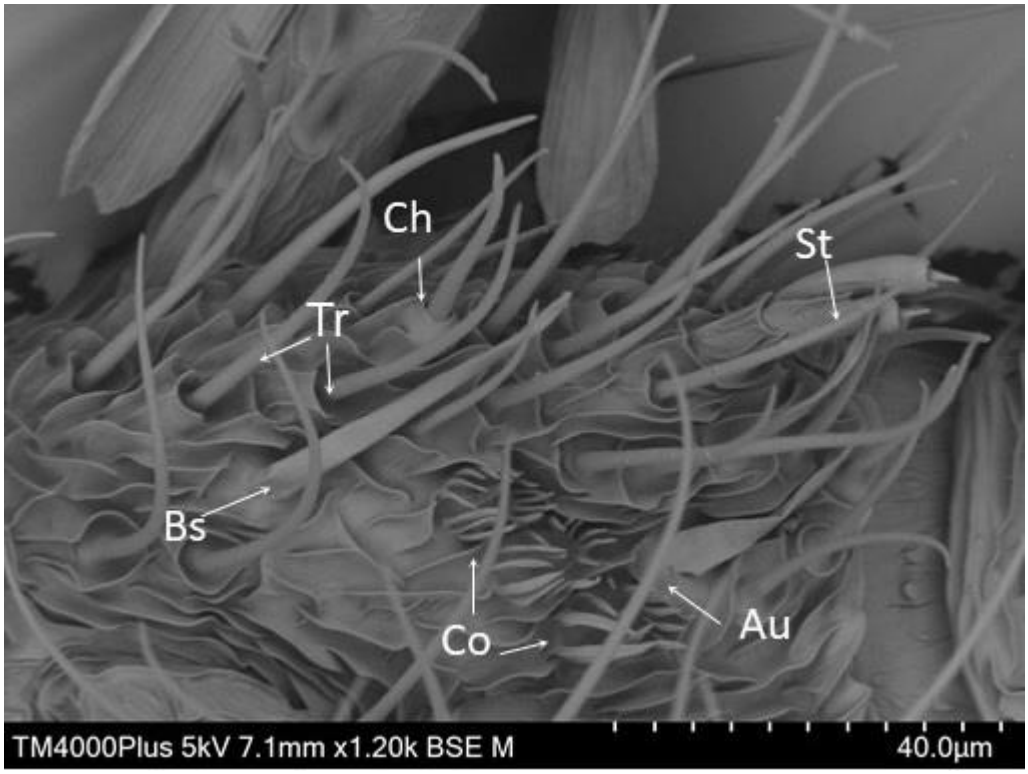

Figure 1. Different types of sensilla located on ventral view on one segment: the long sensilla trichodea (Tr), were the dominant type in a segment; sensilla chaetica (Ch) are similar to Tr but the basal socket is different; sensilla auricillica (Au) have flat shape and are wide at the base; sensilla coeloconia (Co) are a grooved peg surrounded by 12-16 spines; and sensilla styloconica (St) are peg-like, set on top of a conical cuticular style found at the distal margin of each flagellomere.

The average sensilla density for each male, based on the density of sensilla tricodea of two flagellomeres (1st and 35th from the antennal tip), was calculated by dividing the total number of Str observed in those two flagellomeres by the total area in µm^2 of those flagellomeres.

Males were then dissected to measure the testes. The testes are a fused spherical organ located in the 6-9th abdominal segments (Johnson et al., 2017a). The testes were removed with microscissors and placed on a microscope slide. The testes were then imaged immediately (to avoid desiccation) with a Sony camera (ILCE-QX1) mounted on an Olympus microscope (SZX16) at x 5 magnification. Images were measured using ImageJ (Schneider et al., 2012). All measurements were conducted blind to the experimental treatment. Male wing length was used as an index of body size and was also measured, as above.

Effect of immune challenge to female's reproductive investment and ovary mass

To assess the effect of immune challenge on female mate attraction investment, and potential fecundity, female pheromonal attractiveness and ovary mass were recorded.

The effect of immune challenge on female pheromone quality was evaluated, using a glass y-maze olfactometer (Fig. 2), by recording male preferences for pheromones produced from females that were given either an immune challenge or a control treatment.

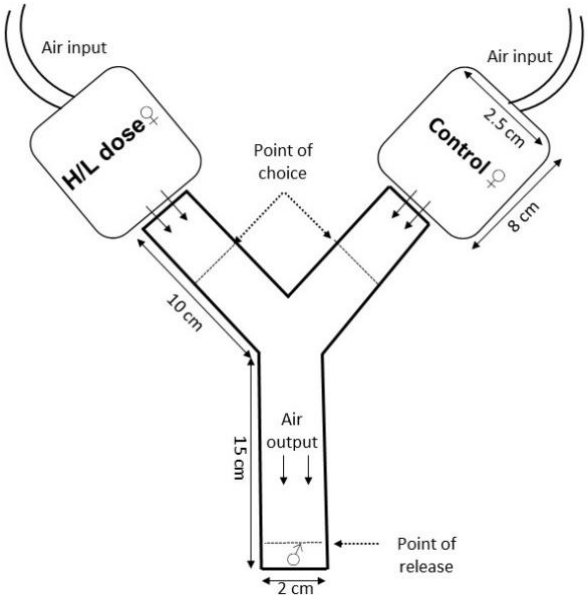

Figure 2. Schematic of the y-maze apparatus used to assess male preferences for sex pheromones from females that received an immune challenge (High (H) or Low (L) dose) or a Control treatment.

Here, a standardized, continuous air flow was introduced at the end of each arm of the y-maze, passing over the females and on to the receiving male. A single, low-dose or high-dose female was placed in one arm and a single control female was placed in the other arm and left to acclimate for one hour. Both females were ≤ 48hrs post eclosion. After both females commenced calling, a virgin, stock male (≤ 36 hrs post eclosion) was introduced into the central arm of the y-maze, and was deemed to have responded when he either walked or flew toward the airflow, travelling at least 5 cm into one of the arms of the y-maze and remaining there for more than 1 min. If males did not make any movement after 30 mins or moved into an arm in less than 10 seconds after their introduction, they were replaced with another male and excluded from further analysis. Males were given 60 min to make a choice, and males that did

not make a choice during this period were excluded from the analysis. Each of the male moths was used once only, and pairs of females were used for one trial only. The olfactometer apparatus was washed with soap and water and dried after each trial. The position of immune challenged females and control treatment was rotated each trial to remove positional effects. The trials were conducted during the middle of the scotophase, when moths are most active (personal observation).

Immediately following completion of the y-maze assays, females were frozen for later ovary mass measurement. The wet weight of the female's ovaries was used as an index of their potential fecundity. Female ovaries were dissected out using micro-scissors from frozen females, all extraneous tissue carefully removed, and then weighed immediately (to avoid desiccation) using a microbalance (Mettler Toledo XS205 Dual range). For all experimental females, wing length was measured as an index of body size (see above for methods).

Statistical Analysis

All statistical analyses were conducted in R studio, version 3.5.2 (R_Core_Team, 2018). The effect of immune challenge treatment on the likelihood of survival until pupation and adult eclosion was explored using a Generalized Linear Model (GLMM - package 'lme4') (Bates et al., 2015), with a binomial error distribution. In these models, relative larva weight (at the time of injection) was used as a covariate. Relative larva weight was calculated separately for each sex as: (individual larva weight—mean larva weight of male or female / standard deviation of larva weight of male or female).

The effect of immune challenge treatment on pupal duration, adult longevity, body size, female ovary mass, male testes size, and sensilla traits were explored using General Linear Models. Where appropriate, relative larva weight (at the time of injection) was used (as above) as males and females larvae differ in weight.

Male olfactory preference trials were analysed using chi-square tests. For every analysis, I examined if males exhibited a directional bias (left or right arm of the olfactometer). However, these were uniformly non-significant, and are not reported.

For all models containing interaction terms, non-significant interactions were removed from final models (Engqvist, 2005). For all General Linear (Mixed) models, I optimally power transformed all dependent variables to maximize normality of model residuals, and the exponents used were noted with every analysis.

RESULTS

In total, 409 ninth-instar and 314 tenth-instar larvae were injected with either an experimental treatment or a control solution. I excluded from the analysis all individuals for which the sex was incorrectly assessed at the juvenile stage (control treatment, n = 16; low dose, n = 17; high dose, n = 22), and all individuals that, despite being sexed correctly, had an additional post-immune challenge molt (control treatment n = 42, low dose n = 13, high dose n = 27).

The effect of immune challenge treatment on the likelihood of survival, longevity and adult body size.

323 males and 263 females were injected and assessed for pre-adult survival. The likelihood of individuals surviving until pupation was affected by an interaction between immune challenge treatment and sex ($\chi^2 = 17.15$, df = 2; $p < 0.001$; Fig. 3). Post hoc tests revealed that the immune challenge dose did not affect the likelihood of male survival to pupation, but that there were significant effects of LPS dose on the likelihood of female survival, with females that received any dose of LPS having a reduced likelihood of survival than females that received a control solution. Finally, relatively heavier larvae were more likely to survive until pupation than their lighter counterparts ($\chi^2 = 27.12$, $\beta = 0.52 \pm 0.11$, df = 1; $p < 0.001$).

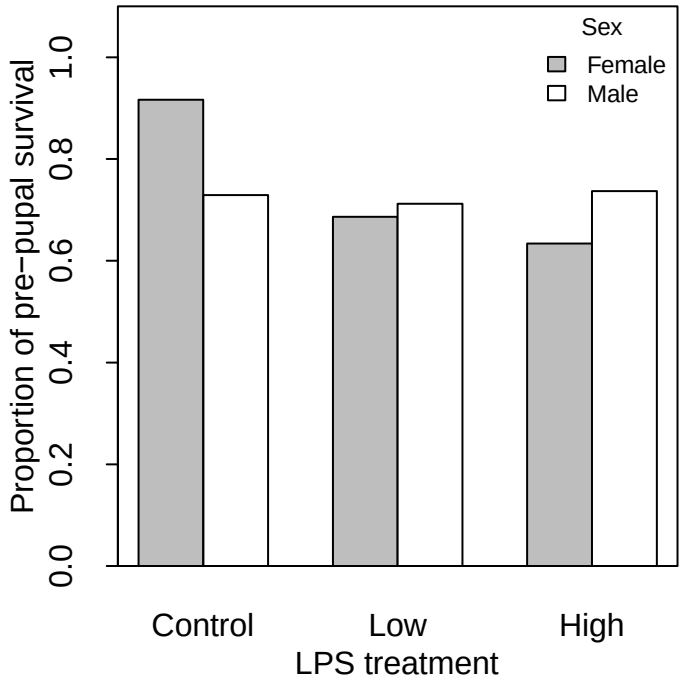

Figure 3. Proportion of individuals from each immune challenge treatment to survive until pupation.

234 ninth-instar and 194 tenth-instar larvae survived until pupation. Of these individuals, the likelihood of survival until adult eclosion was affected by an interaction between immune challenge treatment and sex (χ^2 = 10.99, df = 2; p = 0.004; Fig. 4). Post-hoc tests revealed that immune challenge dose did not affect the likelihood of male survival to adult eclosion, but that there were significant effects of LPS dose on the likelihood of female survival, with females that received a high dose of LPS having a reduced likelihood of survival than females that received a control solution. Finally, the likelihood of surviving until adult eclosion was not affected by larval weight (χ^2 = 0.43, β = -0.12 ± 0.18, df = 1; p = 0.51).

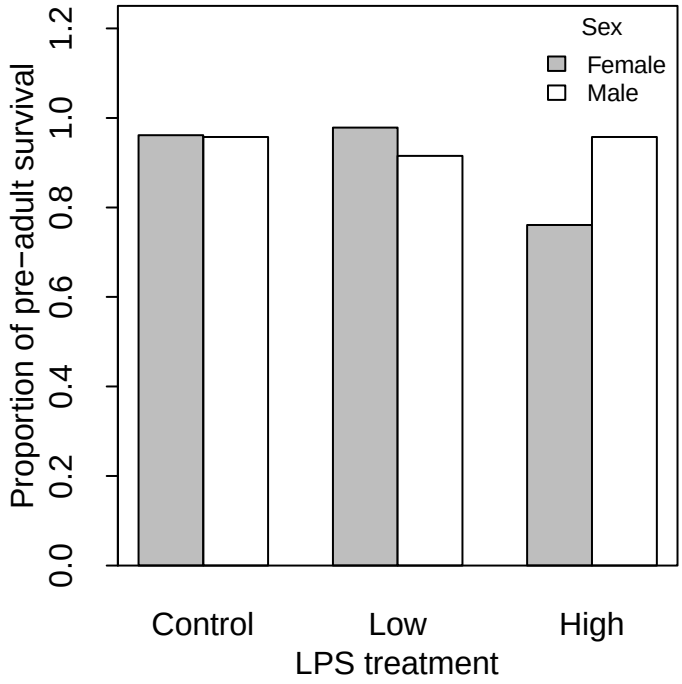

Figure 3. Mean pre-adult survival ± SE of male and female that juvenile received immune challenge: Low, High and Control treatment.

Pupal duration, adult longevity and wing length were measured for 115 males and 92 females (low = 49 males and 25 females, high = 32 males and 28 females; control = 34 males and 39 females).

Pupal duration (raised to the exponent 0.44) was affected by an interaction between immune challenge treatment and sex ($F_{2,200} = 3.62$, $p = 0.03$; Fig. 5). Post-hoc tests revealed that males had longer pupal durations than females, irrespective of their immune challenge treatment (Fig. 5). And while there was no effect of immune challenge treatment on female pupal duration, males that received a low dose of LPS had a shorter pupal duration than males that received either a high dose of LPS or a control solution (Fig. 5). Pupal duration, however, was not affected by the weight of the larva at the time of injection ($F_{1,200} = 1.95$, $\beta = -0.01 \pm 0.007$, $p = 0.17$).

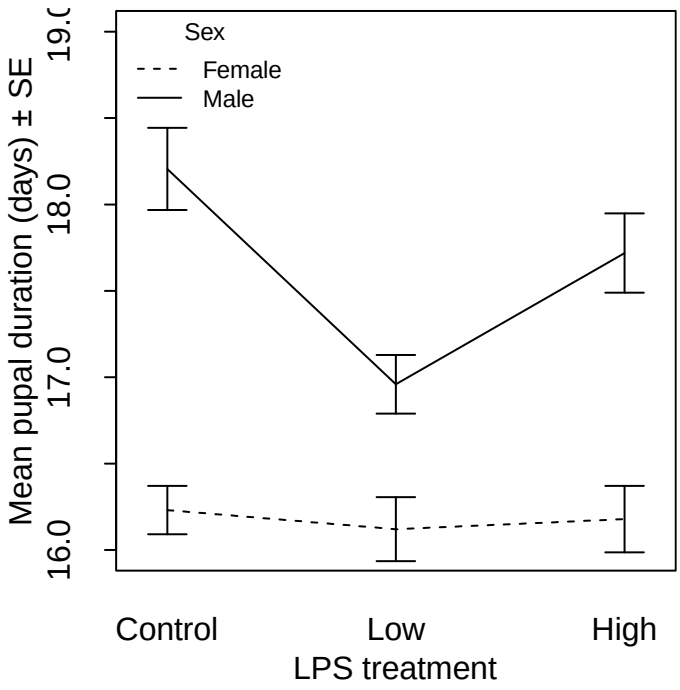

Figure 5. Mean pupal duration ± SE of male and female that juvenile received immune challenge: Low, High and Control treatment.

Male adult longevity (raised to the exponent 1.36) was affected by the immune challenge treatment ($F_{2,111}$ = 3.84, p = 0.02; Fig. 6). Post-hoc analysis revealed that males that received a high dose of LPS lived longer as adults than males that received a low dose but not males that received a control solution. Male longevity, however, was not affected by his weight as a larva at injection ($F_{1,111}$ = 0.20, β = -0.35 ± 0.71, p = 0.62).

Adult female longevity (raised to the exponent 0.52) was also affected by the immune challenge treatment ($F_{2,88}$ = 5.09, p = 0.008; Fig. 6). Post-hoc analysis revealed that females that received LPS, irrespective of the dose, lived longer than females that received a control solution. Female longevity, however, was not affected by her weight as a larva at injection ($F_{1,88}$ = 0.03, β = 0.0006 ± 0.04, p = 0.82).

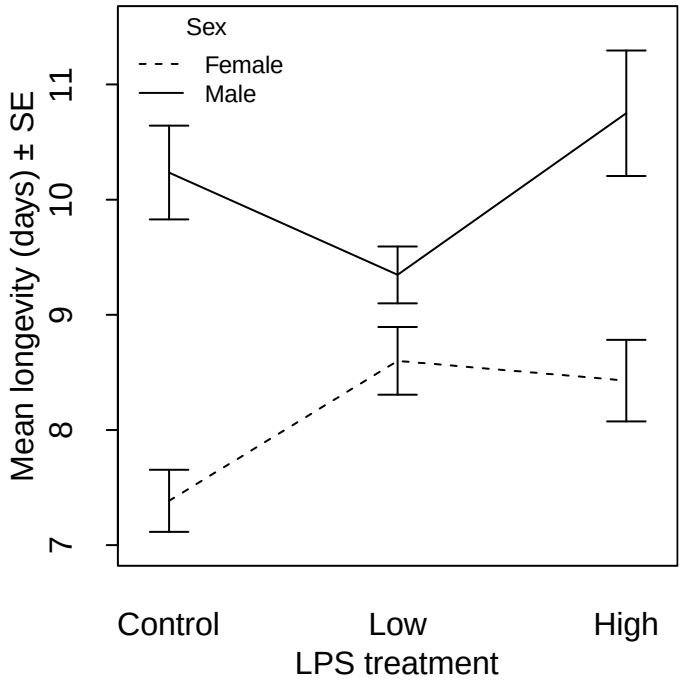

Figure 6. Mean adult longevity ± SE of males and females from each immune challenge treatment.

Wing length (raised to the exponent 1.84) was not affected by immune challenge treatment ($F_{2,202}$= 2.62, p = 0.08). However, males were smaller than females (mean ± standard error wing length for females = 10.45 ± 0.05; for males = 7.79 ± 0.04; $F_{1,202}$ = 1872.41, β = -31.21 ± 0.72, p < 0.001). Finally, larger larvae eclosed into larger adults ($F_{1,202}$ = 9.57, β = 1.10 ± 0.36, p = 0.002). A non-significant interaction between immune challenge treatment and sex was removed from final model ($F_{2,200}$ = 2.99, p = 0.05).

Juvenile immunity-dependent male's pre- and post-copulatory reproductive investment

Sixty males (high = 31; control = 29) were used to measure testes size and antennal morphology.

Testes size (raised to the exponent 1.2) was affected by an interaction between the immune challenge treatment and the weight of the larva at injection ($F_{1,55}$ = 6.34, p = 0.02; Fig. 7). This significant effect was driven by a positive relationship between larva weight at the time of injection and testes area for males that received a control solution, but not for males that received a high dose of LPS.

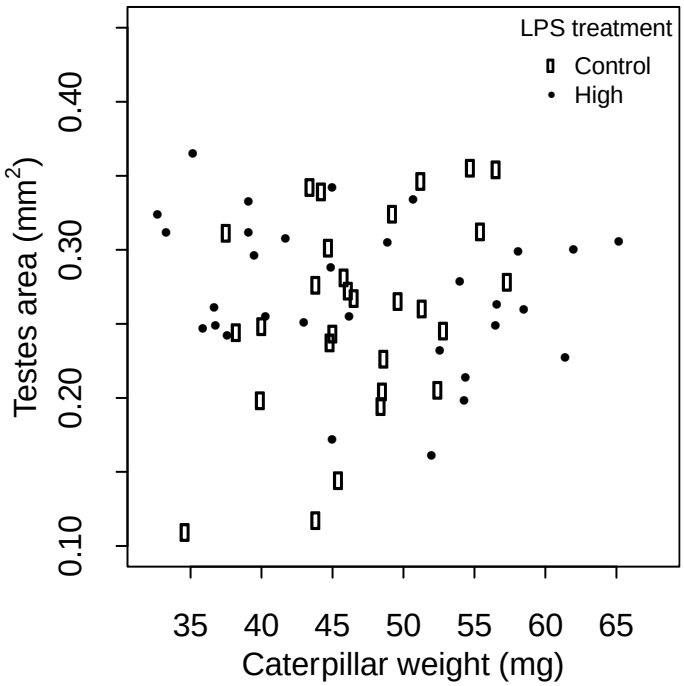

Figure 7. The relationship between male testes size and caterpillar weight at injection for males that received a high dose of LPS or a control solution.

Antennae length (raised to the exponent 4.0) was not affected by the immune challenge treatment (mean ± standard error antennal length: high = 5.675 ± 0.035; control = 5.679 ± 0.043; $F_{1,57}$ = 0.00, p = 0.99) nor by the weight of the larva at injection ($F_{1,57}$ = 0.84, β = 2.37 ± 2.58, p = 0.36). A non-significant interaction between immune challenge treatment and larval weight was removed from the final model ($F_{1,56}$ = 0.19, p = 0.66).

The number of flagellomeres (raised to the exponent 0.04) was not affected by either the immune challenge treatment (mean ± standard error number of flagellomeres: high =

49.52 ± 0.49; control = 49.59 ± 0.51; $F_{1,57}$ = 0.09, p = 0.92) or by the weight of the larva at injection ($F_{1,57}$ = 0.02, β = -5.66e-06 ± 4.31e-05, p = 0.90). A non-significant interaction between immune treatment and larval weight was removed from the final model ($F_{1,56}$ = 3.92, p = 0.05).

Sensilla density (raised to the exponent 0.28) was affected by the immune challenge treatment ($F_{1,57}$ = 4.69, β = -0.003 ± 0.001, p = 0.04; Fig. 8). Post-hoc tests revealed that males that received a high dose of LPS had a lower sensilla density than males that received a control solution (Fig. 8). Sensilla density was not, however affected by the weight of the larva at injection ($F_{1,57}$ = 0.2, β = -4.30e-05 ± 9.30e-05, p = 0.65). A non-significant interaction between immune challenge treatment and larva weight was removed from the final model ($F_{1,56}$ = 0.0009, p = 0.98).

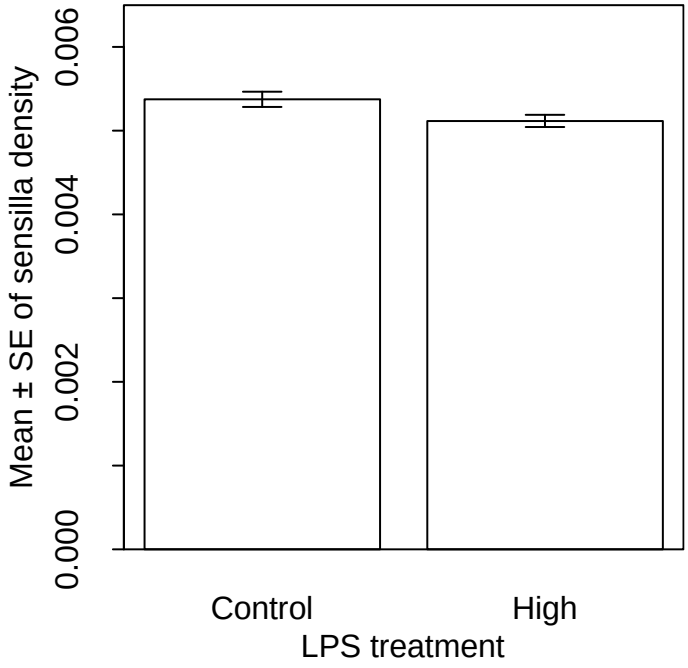

Figure 8. Mean ± standard error sensilla (Str) density of male females received a High dose immune challenge or a Control treatment.

The effect of immune challenge on female pheromonal attractiveness

Thirty-two males (from 38 trails) made a successful choice between females that received a high dose of LPS or females that received a control solution. Twenty (62%) of the males preferred the odour from control, non-immune challenged females, but this did not differ significantly from chance (χ^2_1 = 2.00, *p* = 0.16).

Thirty males (from 35 trails) made a successful choice between females that received a low dose of LPS or females that received a control solution. Males also showed no preference between the pheromones produced by females that received a low dose of LPS and control females (low dose = 19; control = 11; $\chi^2_1 = 2.13$, $p = 0.14$).

The effect of immune challenge on female ovary mass

Ovary mass was measured in 75 females (low = 22, high = 24; control = 29). Ovary mass (raised to the exponent 0.68) was smaller for females that received a low dose (but not a high dose) of LPS, compared to a control solution ($F_{2,71} = 2.93$, $p = 0.06$; Fig. 9) and for individuals that were smaller at the time of injection ($F_{1,71} = 18.63$, $\beta = 0.05 \pm 0.01$, $p < 0.001$). A non-significant interaction between immune challenge treatment and caterpillar weight was removed from the final model ($F_{2,69} = 2.91$, $\beta = 1.23 \pm 0.28$, $p = 0.06$).

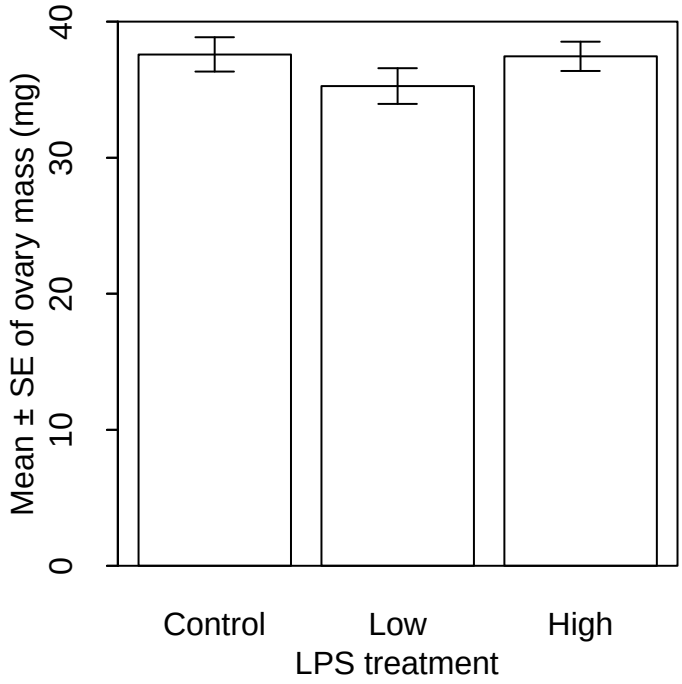

Figure 9. Mean ± standard error ovary mass of females received an immune challenge (Low or High dose) or a Control treatment.

DISCUSSION

My experiments reveal that experimental manipulation of immune investment has impacts on pre- and post-copulatory reproductive investment and life-history traits in both male and

female gumleaf skeletonizer moths, although not in a consistently dose-dependent manner. I demonstrate, for the first time, trade-offs affecting male sensory receiving structures, highlighting the costs of elaborate antennae, and their condition-dependent role in mate location. Males balanced this reduced investment into antennal structures with investment in other life-history traits: males that received a high-dose of LPS had a longer pre-adult period, and a greater longevity. For females, I found no evidence of immune activation costs on pheromone investment, as males were equally attracted to females regardless of immune status. However, there were clear fecundity costs to immune activation in this system. Additionally, immune activation affected female life-history traits, with females living longer. Together, these results highlight the differential costs paid by signallers (females) and receivers (males) in a chemical signalling system.

Juvenile immune challenge has a significant impact on male mate searching in this species, affecting both male investment into functional antennal morphology and their longevity or timeframe in which they are able to find receptive females. Antennal structures are costly to produce (Elgar et al., 2019). The chemoreceptor, tricodea sensilla, for instance, has a number of pores on the surface which function as chemical receiver micro-organs (Hansson, 1995; Shields, 2005; Triseleva & Safonkin, 2006). These structures require significant and costly neural innervation (Niven & Laughlin, 2008; Sanes & Hildebrand, 1976; Stockl et al., 2016). Although the length of the antennae was not different, the reduced density of sensilla produced by immune challenged males suggests that the capacity for pheromone detection is weaker in these males (Gill et al., 2013), which is likely to disadvantage their mate searching success (Elgar et al., 2019; Jayaweera & Barry, 2017; Johnson et al., 2017b). For example, male false garden mantids, *Pseudomantis albofimbriata,* that have a greater density of sensilla trichodea on their antennae arrived at calling females more quickly (Jayaweera & Barry, 2017). Similarly, the density of sensilla plays an important role in efficiency of chemical communication in the weaver ant, *Oecophylla smaragdina* (Gill et al., 2013). Yet, I also found some evidence that this reduced male investment into chemical detection of females may be balanced by increased longevity, as males that received a high dose of LPS had greater longevity than males that received a low dose, but not that of control males.

There is evidence that pheromone production in both males (Chemnitz et al., 2017; Rantala et al., 2003a; Rantala et al., 2003b; Sadd et al., 2006; Vainikka et al., 2007; Worden et al., 2000; Worden & Parker, 2005) and females (Barthel et al., 2015; Harari et al., 2011; van

Dongen et al., 1998) is a costly, condition-dependent trait, and that the expression of this sexual signal can trade-off with investment in immunity (Barthel et al., 2015; Rantala et al., 2003a; Sadd et al., 2006; Vainikka et al., 2007; Worden et al., 2000; Worden & Parker, 2005). For example, the profile of sex pheromones produced by bacteria-injected female tobacco budworm moth, *Heliothis virescens*, was altered, resulting in immune-challenged females having a reduced mating success (Barthel et al., 2015). Similarly, an immune challenge reduced male pheromonal attractiveness to females in the grain beetle *Tenebrio molitor* (Worden et al., 2000; Worden & Parker, 2005). The failure of an immune challenge to elicit a comparable reduction in pheromone investment is surprising, given that I have also demonstrated that female pheromone quality in *U. lugens* is modulated in response to other factors. Instead, my results suggest that the quality and/or quantity of sex pheromone produced was not different. Similarly, immunocompetence of male burying beetles, *Nicrophorus vespilloides*, had no influence on their sex pheromone profile and their attractiveness (Chemnitz et al., 2017). Perhaps, female *U. lugens* prioritise investment into pheromones (at the expense of gamete investment). Female mating failure is expected to be higher in species with low female mating rates and immobile females (Rhainds, 2019), and so avoiding mating failure may be at a premium for *U. lugens*. Alternatively, trade-offs between immune function and reproduction may, in some cases, be evident only under resource stress (Simmons, 2012), such as nutrient limitation.

There is increasing evidence that immune challenges can also affect post-copulatory investment. For example, male decorated crickets, *Gryllodes sigillatus* injected with LPS reduced their spermatophore size (Kerr et al., 2010). The sperm viability of Pacific field crickets, *Teleogryllus oceanicus,* is reduced with immune challenge (Simmons, 2012), and cotton bollworms, *Helicoverpa armigera*, when immune challenged as juveniles, produced fewer sperm (McNamara et al., 2013a). My study, however, shows equivocal evidence of trade-offs with male post-copulatory traits: there was no main effect of immune treatment on testes size, instead there was a positive relationship between body weight and testes area for unchallenged males, but not for immune-challenged males. This equivocal evidence contrasts with the dose-dependent reduction in ovary mass in immune-challenged female *U. lugens*. Egg production is a highly resource-dependent trait, and the trade-offs that are incurred from immune challenges may be especially pronounced in capital breeders, such as *U. lugens*, where individuals acquire all their resources for reproduction in the juvenile stage. Several empirical

studies have revealed phenotypic trade-offs in fecundity following immune challenges (Ahmed et al., 2002; Hurd, 2001). Similar to my study, immune activation has a negative impact on ovary mass in the mosquito, *Anopheles gambie* (Ahmed et al., 2002). However, such trade-offs are not always found. For instance, the egg production of a cricket, *Acheta domesticus*, increased following exposure to a pathogenic bacteria (Adamo, 1999) suggesting evidence of female 'terminal investment'. Alternatively, immune challenge may also have no demonstrable impact on female reproductive output: the egg production of *H. armigera* was not affected following injection with LPS (McNamara et al., 2013a). The equivocal nature of these trade-offs suggest that this relationship is likely to be species-specific and may also depend on the type of immune challenge that is used.

The high reproductive costs paid by males in this mating system, evidenced by their lengthy matings and the transfer of large spermatophores, coupled with the low mating frequency of females, may have affected the trajectory of development in immune challenged individuals. Unlike many animal species in which males can mate multiple times per day, *U. lugens* males and females can only mate once a day. Thus, the number of matings is not only limited in females, but also in males. In such species, a male's capacity to increase his mating frequency is coupled to his longevity. Thus, in *U. lugens*, males and females can be expected to invest into immunity. Males challenged with LPS had a reduced pupal duration and lived longer than males that received a low LPS dose. This result contrasts with other species, such as male cotton bollworm moths, *Helicoverpa armigera*, which had shorter pupal durations following an immune challenge (McNamara et al., 2013a). Female *U. lugen*s also showed increased longevity following immune challenge, but this was balanced by a greater mortality at the pre-adult stage. The fact that female, but not male, mortality increased after receiving LPS suggests a sexual dimorphism in constitutive immunity in this species, with females having a lower immunity. While this is the opposite of predictions following traditional Bateman gradients of immunity (Rolff, 2002), this may reflect the significant male reproductive investment in this species, as has been reported in other moth species with comparable life-histories (Barthel et al., 2015). The degree of dimorphism in constitutive and expressed immunity should be assessed in *U. lugens*. Quantification of these traits may allow greater understanding of the selective pressure that shape immune responses in this species.

This study demonstrates the trade-offs between immune response and pre- and post-copulatory reproductive investment in males and females. Immune challenges affect the sexes

differently, with immune challenges affecting the mate searching capacity of the male (sensilla density and longevity), but not post-copulatory investment. Conversely, females maintained their investment into chemical signalling, instead reducing their reproductive output in response to immune challenge. These differences are likely to reflect the relative importance of pre-and post-copulatory selective pressures on these sexes in this species.

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