Evolution of sociality in insects: insights from the only known social colletid bee *Amphylaeus morosus*

Lucas Hearn

Bachelor of Science (Honours)

Thesis
Submitted to Flinders University
for the degree of Doctor of Philosophy

Flinders University of South Australia
College of Science and Engineering
9th of August 2022

Table of Contents

Thesis Abstract

Understanding how social behaviour first evolves requires that we can identify species that have only recently evolved social traits. I investigated the transition from solitary to social living using the only unambiguously known social species in the bee family Colletidae, *Amphylaeus morosus* (Smith, 1879).

Ecological factors such as parasitism and predation have been emphasised as important facilitators promoting cooperative nesting. Here I detail the host-parasite associations of *A. morosus* with eight different parasitoid species in the Dandenong Ranges, Victoria, and how these parasitoids may have influenced social nesting dynamics of their host. Targeted sampling of *A. morosus* nests across the reproductive season revealed that these eight parasitoid species have staggered timings of attack. I found that as the reproductive season progressed, the number of host adults in a nest declined, often to zero, but the presence of even one adult host female during late brood-rearing stages appeared to offer substantial brood protection against mutillid wasps. These temporal patterns in parasitism and host colony size mean that the benefits of initial colony size may not be evident until much later in the reproductive season as adult colony members gradually die, leading to a temporal dissonance between early and late stages of the reproductive season. Understanding these relationships may provide insights into social evolution that have not been previously explored in studies that take temporal ‘snapshot’ measures of how group size affects colony productivity.

Using genome-wide SNP genotyping, I inferred robust pedigree relationships to identify maternity of brood and intracolony relatedness for colonies of *A. morosus* at the end of the reproductive season. I show that social behaviour in this species involves the formation of both matrifilial and full-sibling colonies with either complete or near-complete monopolization of reproduction in absence of morphological hierarchies. My results suggest that secondary females gain large indirect fitness benefits from any defensive outcomes and these benefits satisfy the conditions of kin selection. These results suggest an avenue to eusociality that involves high relatedness and, very surprisingly, extreme reproductive skew in its earliest stages.

Finally, I show that sex allocation in this species is defined by split sex ratios between solitary and social nests. Socially nesting mothers produced more sons when a non-reproductive nest guard remained in the natal nest whilst solitary foundresses biased their investment towards daughters. These results provide evidence that population sex ratios are indirectly influenced by decisions by some females to become guards although guards remaining in their natal nest are not able to actively manipulate sex ratios in their favour. This pattern of male biased allocation in social nests could raise the threshold for social nesting to spread throughout the population, which contrasts previous views of split sex ratios.

This thesis demonstrates that sociality in *A. morosus* is strongly driven by defence against parasitism. Social behaviour in this species is consistent with kin selection theory, but this form of social nesting does not appear to promote a high frequency of social colonies. These findings challenge previous notions of the major transition from solitary to social living in insects.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Lucas Robert Hearn

List of Contributors

Recipient of RTP support and stipend from the Australian Government Research Training Program Scholarship.

Research and conference grants awarded:

- Holsworth Wildlife Research Endowment, 2020 and 2021
- Flinders College of Science and Engineering, Conference Travel Grant, 2019
- Australian Entomology Society Student Travel Grant 2019
- IUSSI Travel Grant 2022

Co-Author Contributions

List of supervisors:

Supervisor	Michael P. Schwarz College of Science and Engineering Flinders University, Australia
Associated Supervisor	Mike G. Gardner College of Science and Engineering Flinders University, Australia
Adjunct Supervisor	Mark I. Stevens South Australian Museum Adelaide, Australia

List of co-authors:

Lucas Hearn – LRH, Michael Schwarz – MPS, Mark Stevens – MIS, Ben Parslow – BAP, Kevin Williams – KAW, Olivia Davies – OKD

Chapter	Concept & design	Planning & implementation	Data collection	Analysis & interpretation	Writing & formatting
1	LRH, MPS	LRH, MPS, MIS	LRH	LRH, MPS, MIS	LRH
2	LRH, BAP	LRH, BAP, MPS, MIS	LRH, BAP	LRH, BAP, KAW	LRH, BAP, KAW, OKD
3	LRH, MPS, BAP, MIS	LRH, MPS, BAP	LRH	LRH, MPS	LRH, MPS, BAP, MIS
4	LRH, MPS, MIS	LRH, MPS	LRH	LRH, MPS	LRH, MPS, BAP, MIS
5	LRH, MPS, OKD	LRH, MPS, OKD	LRH, OKD	LRH, MPS	LRH, MPS
6	LRH, MPS, MIS	LRH	LRH	LRH, MPS, MIS	LRH, MPS, MIS

List of Original Publications and Conference Presentations

Publications:

- Stevens MI, Matthews CS, Parslow BA, Fagan-Jeffries EP, Dorey JD, Davies OK, Holder J, Young M, Velasco-Castrillion A, **Hearn LR**, Groom SVC, Austin AD, Schwarz MP (2019). Hidden secrets of biodiversity: DNA barcoding of Hymenoptera suggests a precision in species richness estimates that does not match accuracy. *Genome*. 62:437. https://doi.org/10.1139/gen-2019-0083
- **Hearn LR**, Williams KA, Stevens MI, Schwarz MP, Davies OK, Parslow BA (2019). Description and novel host records for a new species of Australian mutillid wasp (Hymenoptera: Mutillidae) from hylaeine bee nests (Hymenoptera: Colletidae). *Austral Entomology*. 58:524–532. https://doi.org/10.1111/aen.12414
- Dorey JB, Rebola CM, Davies OK, Prendergast KS, Parslow BA, Hogendoorn K, Leijs R, **Hearn LR**, Leitch EJ, O'Reilly RL, Marsh J, Woinarski JCZ, Caddy-Retalic S (2021). Continental risk assessment for understudied taxa post-catastrophic wildfire indicates severe impacts on the Australian bee fauna. *Global Change Biology*. 27:6551–6567. https://doi.org/10.1111/gcb.15879
- **Hearn LR**, Stevens MI, Schwarz MP, Parslow BA (2021). Parasitoids of the uniquely social colletid bee *Amphylaeus morosus* (Hymenoptera: Colletidae) in Victoria. *Memoirs of Museum Victoria*. 80:183–191. 10.24199/j.mmv.2021.80.10
- **Hearn LR**, Parslow BA, Stevens MI, Schwarz MP (2022). Temporal dissonance between group size and its benefits requires whole-of-lifecycle measurements. *Behavioral Ecology*. 33:606–614. https://doi.org/10.1093/beheco/arac025
- **Hearn LR**, Davies OK, Schwarz MP (2022). Extreme reproductive skew at the dawn of sociality is consistent with inclusive fitness theory but problematic for routes to eusociality. *Proceedings of the Royal Society B*. 20220652. https://doi.org/10.1098/rspb.2022.0652

Conference presentations:

- **Hearn, LR,** Schwarz, MP, Stevens, MI, Gardner, MG, Davies, OK; Insights into early social evolution from the only known social bee in the mega-diverse family Colletidae. Proceedings of the Australasian Systematic Botany Society & Society of Australian Systematic Biologists Conference, Nov 27-29, 2017, Adelaide, South Australia.
- **Hearn LR,** Davies OK, Parslow BA, Stevens MI, Schwarz MP. Extreme parasitism in a social bee: using a whole-of-life-cycle approach to assess selection dynamics at different stages of a bee's life-cycle. Australian Entomology Society, Society of Australian Systematic Biologists & Australian Arachnological Society Scientific Conference, Dec 01-04, 2019, Brisbane, Queensland.
- **Hearn LR,** Davies OK, Schwarz MP. Reproductive skew in the uniquely social colletid bee *Amphylaeus morosus*. Australian Native Bee Conference, Dec 05-07, 2019, Brisbane, Queensland.

    - Australian Native Bee Genomics Working Group
- **Hearn LR,** Parslow BP, Stevens MI, Schwarz MP. Temporal variation in parasite pressure: niche partitioning of seven parasitoids driven by host resource utilization. International Society of Hymenopterists Conference, Nov 12, 2020, virtual symposia.
    - Runner-up best student presentation
- **Hearn LR,** Davies OK, Schwarz MP. Reproductive skew in the uniquely social colletid bee *Amphylaeus morosus*. Australasian Evolution Society Conference, Dec 03-04, 2020, virtual symposia.
- **Hearn LR,** Davies OK, Schwarz MP. Reproductive skew in the uniquely social colletid bee *Amphylaeus morosus*. Animal Behaviour Society Conference, Aug 02-05, 2021, virtual symposia.
- **Hearn LR,** Parslow BP, Stevens MI, Schwarz MP. Variation in parasitism of a stem-nesting bee suggests benefits of sociality vary across the life-cycle. Flinders University Ecology, Evolution and Environment Conference, Nov 19, 2021, poster.
- **Hearn LR,** Parslow BP, Stevens MI, Schwarz MP. Variation in parasitism of a stem-nesting bee suggests benefits of sociality vary across the life-cycle. Ecological Society of Australia Conference, Nov 22-26, 2021, virtual symposia.
- **Hearn LR,** Davies OK, Schwarz MP. Extreme reproductive skew in a primitively social bee provides insights into the earliest stages of social evolution in insects. Australian Entomology Society & Society of Australian Systematic Biologists Scientific Conference, Dec 06-10, 2021, virtual symposia.
    - IUSSI best student presentation

General Acknowledgements

First and foremost, I would like to thank my principal supervisor, Mike Schwarz for introducing me to the weird and wonderful bee, *Amphylaeus morosus*. But most importantly for teaching me to take an abstract look at science. I am truly grateful for the experience and guidance he provided. Secondly, I would like to thank my co-supervisors, Mark Stevens for his advice and eye for detail which greatly improved my work, and Mike Gardner for his support during my candidature. Thank you to my internal committee, Associate Professor's Luciana Moller and Charlie Huveneers for their helpful input and suggestions throughout my PhD research.

Thank you to everyone who went through the LEGS lab during my PhD, I am grateful for your support and advice. Particularly, Ben Parslow for his guidance and enthusiasm on all thing's parasitoids and general life advice, Olivia Davies for her extensive wisdom on all things *Amphylaeus,* teaching and general scientific queries, James Dorey for the use of many amazing images throughout my PhD and a consistently positive attitude, Justin Holder who helped with fieldwork early on and was always willing to offer help in times of need and, Robbie O'Reilly for the many rubber-band wars and coffee chats that kept me going. I am also very thankful to Patricia Slattery and Annie De Stefano who saw out the last push of my thesis and provided many well-timed coffee and lunch breaks.

I would like to thank all of my family and friends for putting up with me during this period. Thank you to my parents, Karyn and Quentin and brother Mitch who have supported me in my pursuit to be a biologist from a very young age. Thanks to my partners parents, Jackie and Gavin for providing many drinks and meals along the way. I am grateful to all my friends, especially Georgia Macaulay and Tim Gowen for both distracting me from my research when I needed it, but also for helping with fieldwork along the journey.

Last but not least, I am incredibly grateful to my partner, Tyla who provided overwhelming support and advice. Be it help with fieldwork, reading drafts or, listening to presentations, I could not have achieved this without her.

General Introduction

The evolution of eusocial insect societies characterised by reproductive skew and, especially, worker sterility has generated substantial debate in the scientific literature due to their ecological dominance and altruistic behaviour which has challenged Darwinian notions of natural selection. While studies of eusocial behaviour have greatly improved our understanding of how worker sterility is maintained (Hamilton 1964*a,b*), a key question of how social behaviour initially evolves and how this translates to effective worker sterility remains unclear.

Understanding how social behaviour first evolves requires that we can identify species that have only recently evolved social traits. To do this requires that we do not look at putative ‘end points’, but rather labile phases of sociality that might represent early steps into sociality, and studies of highly social organisations may not provide those insights into the earliest stages of social evolution. Therefore, attention should be directed toward groups that demonstrate a wide range of social behaviours. Social Hymenoptera represent a unique group in this regard as their diverse spectrum of behaviours and haplodiploidy driven asymmetrical relatedness could indicate certain traits that favour the evolution of cooperative societies (Rehan and Toth 2015).

However, studies on ‘primitively’ eusocial hymenopteran species by numerous researchers over the last six decades have disproportionately swayed our understanding of how simple forms of sociality are structured (Danforth 2002; Gadagkar 1990; Gibbs et al. 2012; Leadbeater et al. 2011; Richards et al. 2003). The problem is that most of those hymenopteran clades have very long histories of prior social behaviour, often going back many tens of millions of years, sometimes even back to Cretaceous times (Rehan et al. 2012), so they do not provide strong insights into the very earliest stages of social evolution.

The short-tongued bee family, Colletidae comprises over 2,500 species and is especially diverse in Australia with approximately 650 species comprising 50% of the endemic bee fauna (Almeida et al. 2011). Globally, colletid bees are recognised as solitary nesters, with the exception of one unique species, *Amphylaeus morosus* (Smith, 1879) (Colletidae: Hylaeinae). *Amphylaeus morosus* has a distribution across south-eastern Australia, ranging from the coastal heathland regions of Tin Can Bay in southern Queensland (Australia) through to the western extent of Portland in south-western Victoria (Australia) (Houston 1969). By far the biggest population of *A. morosus* is throughout the Dandenong Ranges in eastern Victoria. Within the montane habitats of the Dandenong Ranges, *A. morosus* nests in abscised fronds of the rough fern tree, *Cyathea australis* R.Br. Domin. Adult females use this substrate to excavate the pithy interior into a linear tunnel and line it with a cellophane-like substance that acts as an antimicrobial and waterproofing agent, a trait common to many colletid bee species (Almeida 2008). *Amphylaeus morosus* is facultatively social, with solitary and social polymorphisms occurring in the same population. Solitary nests are most common and will contain only a single adult female that construct and provision brood cells alone. In contrast, social colonies can contain up to three adult females, but most commonly consist of two nestmates that were previously shown to be only weakly related ($r \approx 0.26$, Spessa et al. 2000). How societies are structured and the ecological and genetic factors driving social nesting in *A. morosus* is still unclear but could have significant implications for understanding the earliest stages of social evolution in insects.

This thesis explores:

i. The different insect parasitoid species that attack *Amphylaeus morosus* nests in the Dandenong Ranges;
ii. How these insect parasitoid species influence colony-level benefits for social nesting across the reproductive season;
iii. Reproductive skew in social *A. morosus* colonies and how this challenges traditional pathways to eusociality;
iv. How guarding roles indirectly influence sex allocation patterns in a facultatively social species and the consequences of that influence on further selection for sociality.

A brief summary of each chapter is given below:

Chapter 1: *Evolution of early-stage insect societies: are there alternatives to standard kin selection paradigms?*

Kin selection has been the predominant paradigm for understanding why related individuals choose to sacrifice their own reproduction and help raise collateral kin. However, recent debates have questioned whether kin selection alone is enough to explain social behaviour. Using case studies of insect species with characteristics of early-stage societies and where kin selection arguments have been disputed, I highlight a number of alternative and under-explored models that may help explain the evolution and maintenance of early-stage insect societies when combined with kin selection models.

This review chapter was largely instigated by an earlier study of *Amphylaeus morosus* (Spessa et al. 2000) that suggested only a weak role for kin selection, due to low intra-colony relatedness and seemingly low benefits for social nesting. Those problems largely dissipated once I was able to more-fully estimate the impacts of parasites and assay relatedness in colonies using genome-wide

SNPs. However, problems in solely relying on kin selection to explain all instances of sociality across multiple taxa means that this chapter remains relevant, so it has been retained in the thesis.

Chapter 2: ***Description and novel host records for a new species of Australian mutillid wasp (Hymenoptera: Mutillidae) from hylaeine bee nests (Hymenoptera: Colletidae)***

I described a new species of mutillid wasp parasitising nests of *Amphylaeus morosus* in the Dandenong Ranges, Victoria, Australia. This species is the most prolific and destructive parasitoid of *A. morosus*, so it is ecologically important for understanding sociality in *A. morosus,* and it represents the first known host-parasitoid association between the diverse and widespread hymenopteran groups Mutillidae and Hylaeinae.

This chapter has been published in *Austral Entomology* https://doi.org/10.1111/aen.12414

Chapter 3: ***Parasitoids of the uniquely social colletid bee Amphylaeus morosus (Hymenoptera: Colletidae) in Victoria***

I discovered that eight different parasitoid species were attacking *Amphylaeus morosus*. Seven of these parasitoid-host associations were novel, including two new species of mutillid wasp, *Ephutomorpha tyla* (described in chapter 2) and *Ephutomorpha* aff. *varipes*, provisionally identified. Using targeted fieldwork across the reproductive season of *A. morosus* over four consecutive years, I found that each of the eight different parasitoid species were attacking their host at different times over the reproductive season and had temporally different eclosion phenologies, which are likely to impact host behaviour.

This chapter has been published in *Memoirs of Museums Victoria* https://doi.org/10.24199/j.mmv.2021.80.10

Chapter 4: ***Temporal dissonance between group size and its benefits requires whole-of-lifecycle measurements***

The benefits of living in groups drive the evolution of sociality, and these benefits could vary across a species' life-cycle. However, there may be experimental problems in linking group size at one time in a life-cycle to benefits that only become apparent later on when group size has changed. I assessed how the benefits of social nesting may change across the life-cycle using *Amphylaeus morosus*, with a heightened risk of parasitism towards the end of the season. I show that the various parasite-mediated pressures across the reproductive season create a disconnect between the apparent benefits gained early in the season when parasite pressure is low and the benefits gained later in the season from having a guard remaining in the nest. This new evolutionary principal was termed 'temporal dissonance' and has significant implications for understanding how social dynamics change throughout a life-cycle while highlighting the importance of targeted sampling methods that take a 'whole-of-life-cycle' approach.

This chapter has been published in *Behavioral Ecology* https://doi.org/10.1093/beheco/arac025

Chapter 5: ***Extreme reproductive skew at the dawn of sociality is consistent with inclusive fitness theory but problematic for routes to eusociality***

The evolution of eusociality and extreme altruism remains a highly controversial topic, with recent studies questioning long-held views about the roles of relatedness and matrifilial colony structures. Using genome-wide SNPs combined with life-history and morphological data of *Amphylaeus morosus*, I found that secondary females, who largely play a guarding role, gained large indirect fitness benefits from taking on this guarding role in the nest and these benefits were consistent with Hamilton's Rule. I show that the earliest stages of social behaviour can involve extreme reproductive skew in the absence of true castes and that the evolution of sociality may be able to skip key 'rungs' in the paradigmatic 'social ladders' that have been proposed as routes to eusociality. I suggest that the evolutionary landscapes underlying origins of extreme altruism may be very different from traditional assumptions.

This chapter has been published to *Proceedings of the Royal Society B* https://doi.org/10.1098/rspb.2022.0652

Chapter 6: ***The decision to guard vicariously drives split sex ratios in a facultatively social bee***

Split sex ratios in social Hymenoptera can arise through a variety of ways and in some cases have been argued to facilitate the evolution of eusocial behaviour if alloparental care is subsequently directed towards full sisters. I show that *Amphylaeus morosus* exhibits split sex ratios between social and solitary colonies within a single population. Solitary nests produce a female-biased sex ratio, sometimes producing female-only broods, while social colonies produce significantly more male offspring in comparison. I show that these male-biased broods in social colonies arise from the benefit of having a designated nest guard that allows provisioning mothers to increase the number of offspring they produce by mitigating the risk of premature mortality. But this increase in male offspring production lessens the payoffs for guards as guards become more common in a population because the value of males declines as the number of males increases. This effect may put a 'selective brake' on the frequency of females opting to take on guarding roles and limit the potential for guarding behaviour to spread. This might help explain why guarding behaviour in *A. morosus* is consistent with Hamilton's Rule but is nevertheless uncommon.

References:

Almeida EAB (2008) Colletidae nesting biology (Hymenoptera: Apoidea). *Apidologie*. 39:16–29.

Almeida EAB, Pie MR, Brady SG, Danforth BN (2012) Biogeography and diversification of colletid bees (Hymenoptera: Colletidae): emerging patterns from the southern end of the world. *Journal of Biogeography*. 39:526–544.

Danforth BN (2002) Evolution of sociality in a primitively eusocial lineage of bees. *Proceedings of the National Academy of Sciences*. 99:286–290.

Gadagkar R (1990) Origin and evolution of eusociality: a perspective from studying primitively eusocial wasps. *Journal of Genetics*. 69:113–125.

Gibbs J, Brady SG, Kanda K, Danforth BN (2012) Phylogeny of halictine bees supports a shared origin of eusociality for *Halictus and Lasioglossum* (Apoidea: Anthophila: Halictidae). *Molecular phylogenetics and evolution*. 65:926–939.

Hamilton WD (1964*a*) The genetical evolution of social behvaiour I. *Journal of Theoretical Biology*. 7:1–16.

Hamilton WD (1964*b*) The genetical evolution of social behvaiour II. *Journal of Theoretical Biology*. 7:17–52.

Houston T (1969) The systematics and biology of Australian Hylaeine bees (Hymenoptera: Colletidae). Ph.D. thesis, University of Queensland, Hertfordshire, St Lucia.

Leadbeater E, Carruthers JM, Green JP, Rosser NS, Field J (2011) Nest inheritance is the missing source of direct fitness in a primitively eusocial insect. *Science*. 333:874–876.

Rehan SM, Leys R, Schwarz MP (2012) A mid-cretaceous origin of sociality in xylocopine bees with only two origins of true worker castes indicates severe barriers to eusociality. *PLOS ONE*. 7:e34690.

Rehan SM, Toth AL (2015) Climbing the social ladder: the molecular evolution of sociality. *Trends Ecology Evolution*. 30:426–433.

Richards MH, von Wettberg EJ, Rutgers AC (2003) A novel social polymorphism in a primitively eusocial bee. *Proceedings of the National Academy of Sciences*. 100:7175–7180.

Spessa A, Schwarz MP, Adams M (2000) Sociality in *Amphylaeus morosus*. *Entomological Society of America*. 93:684–692.

Chapter 1 - Evolution of early-stage insect societies: are there alternatives to standard kin selection paradigms?

Lucas R. Hearn[1*], Mark I. Stevens[2, 3] and Michael P. Schwarz[1]

[1]*College of Science and Engineering, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia.*

[2]*Earth and Biological Sciences, South Australian Museum, GPO Box 234, Adelaide, SA 5001, Australia.*

[3]*School of Biological Sciences, University of Adelaide, SA 5005, Australia.*

Corresponding author: Lucashearn7@gmail.com*

1.1 Abstract

Kin selection has been the predominant paradigm for understanding social evolution, particularly in insects. However, recent debates have questioned whether it is sufficient to explain social behaviour in complex eusocial societies, so-called superorganisms. One issue that has led to such inconclusive debates is that they have not explored kin selection theory for understanding the very earliest steps in social evolution. If kin selection has difficulties in explaining very simple forms of sociality it may signal a lack of utility across the full range of social structures. We review a number of case studies on species with various forms of sociality, identifying where kin selection arguments are problematic, and suggest alternative selection modes that may be confused with kin selection or act in concert with kin selection. We argue that inferring a role for kin selection must move beyond a default assumption that relatedness within social groups implies a key role for kin selection. Allee effects, reproductive queuing and group augmentation can all give rise to patterns that appear to support kin selection, but understanding their roles can require very difficult longitudinal studies. We identify a variety of model organisms that could be used to disentangle these different forms of selection, and suggest how future studies could best discriminate between these selection alternatives.

Key words: Kin selection, Social evolution, Major evolutionary transitions, Hymenoptera, Thrips

1.2 Introduction

Conditions favouring the evolution of sociality have intrigued evolutionary biologists since *The Origin of Species* was first published (Darwin 1859). The majority of attention has been directed at the origins of eusociality, where social behaviour is characterized by non-reproductive castes assisting the reproduction efforts of kin (Batra 1966). This form of extreme altruism has led to contentious debate surrounding the conditions in which non-reproductive helpers can evolve (Abbot et al. 2011; Boomsma 2009; Gardner and Grafen 2009; Nowak et al. 2010). Attention has largely focused on the factors that have driven the evolution of social nesting from previously solitary behaviour. Wilson and Hölldobler (2005) have argued that the origins of social behaviour derive from a set of 'necessary' preconditions that de-emphasized the role of high genetic relatedness that initially followed Hamilton's (1964*a*,*b*) key papers on inclusive fitness. These preconditions claim that eusocial behaviour cannot evolve without: (i) overlapping generations, (ii) the ability for a nest containing food resources to be defended, and (iii) the potential for cooperation between kin (Crespi 1994; Wilson 2008). In saying that, the majority of models exploring the origins of social behaviour have been developed around the notion that high relatedness is an important mechanism underlying the evolution of cooperation (Foster et al. 2006).

To date, kin selection theory has been the main paradigm for explaining the evolution of complex eusocial societies and so-called 'superorganisms'. First proposed by J.B.S. Haldane in 1955, kin selection theory gained significant traction when Hamilton (1964) expanded it into a mathematical formulation known as Hamilton's rule that stated selection will favour an altruistic allele when the condition $b.r > c$ is met, where the beneficiary of help b, multiplied by the helpers relatedness to beneficiary r, is greater than the direct fitness cost to the helper c. Hamilton's rule is rightfully lauded for its simplicity in creating a framework that allows the explanation of why an individual might forgo reproduction in the presence of kin without the need for complicated mathematical models.

Recent debates have questioned whether kin selection is sufficient to explain social behaviour in sophisticated societies (Fletcher and Doebeli 2009; Fletcher et al. 2006; Gadagkar 2016). However, these debates have not thoroughly explored its utility for understanding the very earliest steps in social evolution. If kin selection has trouble explaining primitive forms of sociality, does it signal a lack of utility across the full range of social structures? In many cases, the default assumption that *high relatedness within social groups implies a key role for kin selection* is not always supported (e.g. Rehan et al. 2014). Furthermore, testing the functionality of kin selection is difficult as it requires quantifiable measures for the costs and benefits of helping acts, which is often only achievable through species that exhibit social and solitary nesting in sympatry (Ohkubo et al. 2018; Richards 2019; Yagi and Hasegawa 2012). There is increasing evidence of social behaviour in insects that involve mutualistic rather than altruistic associations (Dew et al. 2016; Kukuk and Sage 1994; Wcislo and Tierney 2009). Mutualistic relationships that lead to increased nest defence, nest inheritance, and increased group fitness benefits are thought to play an important role in the initial evolution of sociality in insects (Crespi 1994; Lin and Michener 1972). Whether these alternative factors act in lieu of kin selection or supplement kin selection is hard to decipher without convoluted longitudinal studies.

Paradigms such as kin selection are imperative for guiding science. They determine what tests should be conducted and how the outcomes of those tests should be interpreted. Equally so, these models should hold up under rigorous theoretical and empirical testing. While kin selection theory is a powerful explanatory tool for exploring the underpinnings of social evolution, it has continually been questioned for its inability to broadly capture selection dynamics in a variety of model systems. The debate for and against kin selection is complex. While it is easy to hold a lack of empirical evidence against kin selection, it is just as easy to make similar arguments for the multiple theories that exist as substitutes for kin selection theory. The uncertainty in recent paradigms of social evolution bring to light a greater issue regarding whether kin selection and its alternatives are truly falsifiable.

To fully understand the role of kin selection in the evolution of simple cooperative societies requires relatedness values paired with behavioural observations. Here we examine a variety of model organisms where intracolony relatedness estimates are able to be combined with behavioural data to give a comprehensive overview of how selection dynamics may be maintaining social behaviour in these select species. We argue that alternative selection modes, including Allee effects, group augmentation, reproductive queuing and the central limit theorem can all give rise to behaviours that may appear to suggest kin selection, but where relatedness may not be a key factor.

1.3 Transitions from solitary to eusocial

The transition from solitary to eusocial behaviour is generally thought to consist of several intermediate stages. Conceptually, one appealing framework for understanding these transitional steps has been the ‘social ladder’ model (Evans and West-Eberhard 1973). This approach assumes species exhibiting simple social forms represent the first stage of a progressive stepwise system striving towards eusociality. In constructing this approach, Evans and West-Eberhard (1973) were quick to clarify that each transitional step represented a stable strategy with its own adaptive value. However, this ladder approach has come under recent scrutiny for its inconsistencies when trying to infer the early stages of eusocial evolution from extant 'ancestors' with simple social traits (Linksvayer and Johnson 2019). For instance, some past studies on species exhibiting apparently 'early' stages of social behaviour have involved species that derived from ancestors where social behaviour was more complex, and so represent 'reversions' to apparently less complex behaviour (Gibbs et al. 2012). The issue that arises is that such behavioural traits do not represent traits similar to those that evolved at the dawn of social evolution but, merely the retention of traits that evolved during periods of social complexity. Linksvayer and Johnson (2019) highlighted the need for caution when using extant species with simple social forms to elucidate the genetic and behavioural underpinnings of ancestral forms of advanced eusociality, suggesting careful phylogenetic approaches should be used to infer ancestral traits. However, many have suggested that social behaviour in some insect lineages is so labile that it can be frequently lost and gained to a point where phylogenetic inference becomes unreliable (Chenoweth et al. 2007; Wcislo and Tierney 2009).

One workaround is studying species where solitary and eusocial behaviours are facultative within the same population. Richards (2019) has highlighted the value of studying socially polymorphic species when investigating the origins of sociality. Facultatively social species that exhibit both social and solitary behaviours provide excellent systems to comparatively test hypotheses concerning the fitness benefits of a social strategy and provide the only systems to empirically apply Hamilton's rule (Rehan et al. 2010). Furthermore, models of social evolution applied to obligately social species are rarely appropriate for facultatively social species, highlighting the necessary distinction and clear terminology that should be made between different social forms.

1.4 Classification of social terminology

Developing consistent terminology across social taxa is an important step for defining social traits and comparability among scientific studies. The classification of social behaviour has been continually evolving for the last century since Wheeler (1928) initially characterised what defined a social insect. Michener (1969) expanded this characterisation into four major social classes: solitary, subsocial, parasocial, and eusocial, with solitary representing a lack of social behaviour and eusocial, initially defined by Batra (1966), representing the most advanced form of social behaviour (Table 1). These definitions have been refined numerous times (Crespi and Yanega 1995; Wilson 1971), however, the four major classes proposed by Michener (1969), with the associated subclasses have remained the most consistently used definitions within the literature. Dew et al. (2016) argued that the current social terminology is outdated and reflects a eusocial biased perspective on social evolution, where hierarchal behaviours are predominant. Instead, the term ‘casteless’ was proposed to define behaviours which lack distinct hierarchies and where reproductive partitioning is egalitarian.

The primary issue associated with classifying social systems is that, in many species, social behaviour is not definitive enough to be grouped under one social form. Rather, species may exhibit a variety of strategies over their lifetime in response to environmental and behavioural conditions. The consistent classification of social terminology is an important step for developing evolutionary models. It allows the grouping of behaviours that are specific to a certain social classification, which can then be modelled to explain the mechanisms underlying the maintenance of sociality for different social brackets.

1.5 The paradigms of social evolution

The predictions of kin selection theory that implies genetic relatedness corresponds to social behaviour, provides a fundamental model for why animals choose to sacrifice direct reproduction to help and insinuates that cooperative societies evolved due to increased direct benefits from helpers, where the indirect fitness an altruist may gain is greater than what it may achieve through a solitary lifestyle (Bourke 2014). This theory allows for testable hypotheses to be developed to explain how cooperative behaviour has evolved through maximising fitness through direct reproduction or assisting kin to reproduce (Queller and Strassmann 1998)

Kin selection theory has not been universally accepted by all social biologists. Wilson and Hölldobler (2005) were the first to argue that genetic relatedness may not be as important as previously emphasised, suggesting that high relatedness may be a consequence of sociality rather than a causative element (Wilson 2008). Nowak et al. (2010) expanded these arguments into what is currently the most high-profile critique of Hamilton’s kin selection theory, arguing that standard natural selection theory provides a better model to explain the evolution of eusociality compared to kin selection theory which “requires stringent assumptions”. Their argument that kin selection requires strict assumptions to operate is controversial for many reasons. For instance, if high relatedness is held as a constant, as argued by Nowak et al. (2010), then kin selection via Hamilton’s rule could only operate on variation in b and c. Rather, Hamilton’s rule requires variation among all parameters (r, b, c) which, in turn, broadens the selection criteria for eusociality to evolve (Liao et al.

2015). While the arguments of Nowak et al. (2010) were swiftly refuted by multiple responses (e.g. Abbot et al. 2011; Boomsma et al. 2011; Gardner et al. 2011; Herre and Wcislo 2011; Strassmann et al. 2011), those responses highlight a greater issue that is yet to be resolved among empiricists and theoreticians alike. Chiefly, whether kin selection is sufficiently broad enough to capture selection dynamics across non-eusocial groups.

While much of the past discussion has focused on critiquing the various mechanisms of sociality, recent discussion has aimed at synthesising the various pathways to social evolution (Gardner et al. 2011). The idea of a universal model such as kin selection being used to explain all facets of social evolution is ideal. In theory, such a model would alleviate the many issues that restrict social evolution studies (Reeve 2001). However, exclusively using one theory to model the complex selection dynamics of social evolution runs the risk of pigeonholing certain evolutionary dynamics. As it stands, the model of kin selection theory assumes that behaviours will continually evolve in a direction that makes the expression of altruistic traits more likely, the more closely related individuals are to each other. There are a number of key cases that do not support this assumption, which prompts the need to find alternative or supplementary explanations for why unrelated individuals nest together or, where the behaviours expressed in highly related societies do not fit the parameters set by kin selection.

1.6 Case studies

Theories modelled around kin selection theory maintain that high relatedness is required for the evolution of social behaviour from solitary ancestors. However, these models are not consistent within the current literature. As it stands, there has been little empirical or theoretical support for upscaling models that apply to early-stage societies to explain the evolution of eusociality.

Subsocial and parasocial bees

Communal behaviour is taxonomically widespread in the Aculeata (Hymenoptera), occurring in many of the major bee and wasp families (Kocher and Paxton 2014; Wcislo and Tierney 2009). However, the biology and evolution of this ‘primitive’ form of sociality is not well known. Characterised by egalitarian associations with flexible nesting dynamics, communality frequently involves unrelated individuals nesting together to enhance direct fitness benefits (Lin and Michener 1972). Subsequently, this means any indirect fitness benefits an individual may gain are unlikely to be high, making the role of kin selection in the evolution of these societies intriguing.

The communal bee, *Lasioglossum (Chilalictus*) *hemichalceum* is one of the few halictine species where non-related individuals consistently nest together. Kukuk et al. (2005) suggested that the low intracolony relatedness in this species is the result of second-generation females initiating nests with unrelated individuals, suggesting a limited role for kin selection as a mechanism promoting cooperative nesting. Why second-generation females choose to co-found nests with unrelated individuals is thought to be via selection to minimize inbreeding (Kukuk et al. 2005). Nests containing unrelated females had been assumed to be egalitarian in nature (Kukuk et al. 1998), supporting the notion that nesting with non-relatives is a stable strategy for this species. However, Kukuk et al. (1998) found that up to 60% of females in a colony of this species did not participate in foraging, an activity that is considered 'risky' in social Hymenoptera (O'Donnell and Jeanne 1992). Why only some individuals partake in foraging is not fully understood. However, it seems that this form of non-kin-based sociality is best explained as a mutualistic association where all group members receive direct fitness benefits and where 'risk taking' foragers receive fitness insurances via the protection of their brood if they die.

The communal bee *Perdita texana* provides another example where nesting with unrelated females is common. *Perdita texana* is a univoltine, facultatively communal bee that exclusively pollinates *Opuntia* spp. (Neff and Danforth 1991). Nests may contain up to 28 females yet, generally contain no more than five reproductively active females at a time. Females either remain in their natal nest or disperse to initiate new nests, in which they are often joined by unrelated females to cooperatively found communal nests. While not much is known about the specifics of sociality in this species, multilocus DNA fingerprinting suggests that it is more common for nests to consist of unrelated individuals compared to half-sisters or full sisters, with mean intracolony relatedness estimated as close to zero (Table 2). Danforth et al. (1996) suggested that cooperative nesting in *P. texana* arises from costs of dispersing to found a new nest alone, arguing that selection will favour re-using a natal nest, joining a pre-existing nest or cofounding a new nest. Adults in this species have short lives, leading to temporal constraints on reproductive opportunities, where time spent constructing a new nest can overlap with the flowering time of *Opuntia* spp. and therefore their ability to amass provisions. Furthermore, it is likely that multifemale nests form mutualistic associations that provide direct fitness benefits via unrelated nestmates helping defend the nest when an individual is out foraging or prematurely dies.

The allodapine bee, *Exoneura nigrescens* forms colonies that can range from a solitary mother and her brood to primitively eusocial colonies of up to eight adult females cooperatively rearing brood in a communal chamber (Schwarz 1987). Females may either nest independently, cofound new nests with related individuals or join nests consisting of unrelated individuals (Silberbauer and Schwarz 1995). This variation in social structure that can see unrelated nestmates cooperating is thought to derive from increased per capita benefits from social nesting. Task specialisation in this species is still unclear although, designated foraging and guard roles have been shown (Stevens et al. 2007; Zammit et al. 2008). The existence of these behavioural roles is thought to be key reason for social nesting in this species as increased task efficiency (e.g. nest defence and provisioning rate) minimises the costs of brood loss through ant predation and under provisioning that is common for independent breeders.

The small carpenter bee, *Ceratina australensis* presents a rare case of Hamilton's rule being empirically tested. *Ceratina australensis* is facultatively social, with females nesting solitarily, or forming colonies of two adult females (Rehan et al. 2010). Sociality in this species, like many other ceratinines, appears to stem from extended maternal care whereby mothers remain in the nest and help rear their daughters to adulthood. However, social nesting primarily arises from two sisters remaining in a natal nest. In this case, non-reproductive, social secondaries are thought to help defend the nest, while also waiting to inherit the nest and lay their own brood. Rehan et al. (2014) found higher observed direct benefits for solitary nesters compared to the combined direct and inclusive fitness benefits for social secondaries, suggesting that solitary nesting should be favoured over social nesting. So why does social nesting persist in this species if social behaviour has detrimental effects to lifetime fitness? Most likely, social primaries tolerate social secondaries for their ability to guard the nest when they are foraging. Conversely, the benefits of remaining in a natal nest as a social secondary provide a chance to inherit the nest, which outweighs the costs of dispersing and risking brood mortality via predation.

The eastern carpenter bee, *Xylocopa virginica* is a well-studied facultatively social species with social nests containing up to five adult females. Social nests have been shown to structure around a tertiary reproductive system (Vickruck and Richards 2018). The dominant individual (primary) in a nest generally encompasses the role of dominant breeder, forager and provisioner alike. Whereas the secondary female acts as a subordinate, next in-line for reproductive duties should the primary female not return to the nest within the same season. Interestingly, the tertiary female appears to do very little except occasionally guard the nest, either by actively blocking the nest entrance, or by passively resting in the entrance, a mechanism that allows them to conserve energy and survive into the next season where they become an active egg-layer (Vickruck and Richards 2018). Within nest relatedness in *X. virginica* is low ($r = 0.16$) and this is thought to be driven by a combination of limited nest resources promoting dispersal from a natal nest to avoid the indirect fitness costs of kin competition (Vickruck and Richards 2021). Instead, females maximise direct fitness by queuing for an opportunity to reproduce and in the case of tertiary females, conserve energy in one season to reproduce in the next.

Primitively eusocial wasps

Most violations of kin selection generally arise from early-stage insect societies. However, primitively eusocial wasps present an exception to this, where their more advanced form of social behaviour and complex behavioural roles appears to provide a mode of selection that falls out of the kin selection paradigm. Primitively eusocial wasps are a remarkably well studied group of insects. They exhibit no morphological differentiation between worker and reproductive castes and generally show significant behavioural flexibility (Gadagkar 1990). Within the primitively eusocial wasps, the subfamily Polistinae provide some of the most tractable systems to explore the evolution of eusociality.

The primitively eusocial paper wasp, *Polistes dominula* is one of the most widely studied social models and represents one of the best examples of non-relatives frequently nesting together in a stable association. In *P. dominula,* mated foundresses initiate new nests in spiring, either solitarily, or with a small group of co-foundresses (Nonacs and Reeve 1995). Co-foundress nests consist of one dominant egg-laying female and several helper subordinates which assist in foraging and provisioning brood cells. In this species, a high proportion of subordinates in a nest may be unrelated to the dominant female. In this circumstance, the dominant female lays all the eggs, while subordinate females help at the nest, limiting the net indirect fitness gain in co-founded nests (Liebert and Starks 2006). This system is unlike most social insect colonies containing unrelated individuals where there is usually little to no reproductive skew. In *P. dominula* however, the queen lays nearly all the brood resulting in extremely high reproductive skew between the dominant queen and the subordinate helpers. Leadbeater et al. (2011) showed that subordinates on average produce more offspring than a single foundress. This is because subordinates have the opportunity to inherit the dominant position in the nest towards the end of the season. The ability to lay few eggs as a subordinate with the chance to inherit the nest and gain large direct fitness benefits provides an explanation for why helpers may join a nest with unrelated individuals. However, it does not account for why subordinates would help rear offspring of an unrelated dominant.

Ropalidia marginata represents another well studied primitively eusocial wasp with flexible behavioural castes. In this species, females can be egg-laying queens or workers and generally have four nesting strategies available to them when they eclose as adults; (1) disperse and found a new nest solitarily, (2) disperse and found a new nest with multiple females, (3) remain in their natal nest as a helper for the entirety of their lives, and (4) remain in their natal nest as a helper for the potential to take over the role of an egg-laying queen (Shakarad and Gadagkar 1997). Due to multiple mating of queens and the ability for any worker to inherit the queen role, multiple matrilines can exist within a single nest. For instance, new queens may be daughters, sisters, nieces, or cousins to other colony members, such that relatedness between workers and brood can often be quite low (r = 0.22 – 0.46; Table 2).

Gadagkar (2016) experimentally assigned female wasps one of three potential roles, as either: a voluntary single foundress, a forced single foundress, or a forced solitary queen. He found that productivity (measured as reproductive output) was significantly less in forced single foundresses compared to the other two roles. He concluded that workers only sacrifice a small reproductive output that they may have otherwise gained as single foundresses. Therefore, the worker strategy should be and is, according to empirical data, favoured over the single foundress strategy.

Through empirical tests of Hamilton’s rule, Gadagkar (2016) showed that *R. marginata* is predisposed to eusociality based on ecological and demographic factors rather than the low pairwise relatedness seen between workers and the brood they help rear. While kin selection seems to have played a role in the evolution of sociality in this species, there are several conflicting phenomena that suggest other forces may be at play. For instance, nest foundation and joining by unrelated individuals, a lack of kin recognition and, cooperation between unrelated nestmates. All these factors are unlikely to be shaped by kin selection, yet persist throughout this species, suggesting the influence of multiple evolutionary forces.

Australian gall-forming thrips

Australian gall-forming thrips present an opportunity to investigate a non-Hymenopteran haplodiploid insect taxon with an independent origin of eusociality to explore the factors that may enable the origins of sociality that entails high levels of reproductive skew (Crespi 1992*b*). Australian gall-forming thrips comprise two different forms of colony development within galls: (i) the ancestral form involves foundress mothers initiating a gall, becoming interred within it, and then producing a clutch of offspring that complete their development within the gall before dispersing; and (ii) a more derived form of colony development occurs when the first clutch produced by a foundress develop into apterous or brachypterous adults (both male and female) with enlarged forelegs (Crespi 1992*a*). These individuals produce a second clutch of eggs within their natal galls and also help defend the gall from kleptoparastic thrips species that kill the gall formers and lay their own eggs.

Initial studies of the thrips with soldiers suggested that females have smaller ovaries than the foundress mother and it was thought that this represented an ‘altruistic’ specialisation for defence at the cost of direct reproduction. Behavioural studies also indicated that soldier morphs are more likely to engage in fights with kleptoparasitic invaders than the foundress mother (Perry et al. 2004). Genetic studies indicated a very high level of intra-gall relatedness, suggesting that kinship may be important for explaining soldier morphs. Furthermore, one study inferred a very high level of intra-gall relatedness at the origin of soldier ‘castes’, suggesting that high intra-group relatedness may be implicated in the origins of eusociality and therefore supporting a role for kin selection in the origin and maintenance of altruism (Chapman et al. 2000).

1.7 Alternative selection models for social evolution

Altruistic helping behaviour is generally directed at kin. However, many cases have shown that it is not categorically restricted to closely related social groups (Queller et al. 2000). In societies where members are not closely related enough for altruistic behaviours to have evolved, is kin selection a sufficient explanatory model, or should we be looking for alternative hypotheses to complement kin selection? Most of the alternative theories that have been developed around the earliest stages of social evolution have largely been ignored for their lack of empirical support (Boomsma and Gawne 2018; Kokko and Johnstone 1999; Kokko and Johnstone 2001; Wenzel and Pickering 1991). However, many of the nesting dynamics present in simple insect societies such as nesting with non-relatives and frequent nest joining appear to give rise to behaviours that may favour some of these alternative models.

Group augmentation and the Allee effect

One explanation for why cooperative groups may form in the absence of relatedness is by individuals actively recruiting helpers which may in turn reciprocate help. Kokko and Johnstone (2001) named this explanation group augmentation (GA), a model that promotes increased direct benefits by enhancing group size and subsequently survival and reproductive effort. The mode of selection inferred by the GA model that implies an equilibrium between group size and fitness payoff is not dissimilar to the conceptual framework of the Allee effect (Courchamp et al. 1999). The Allee effect infers a positive correlation between population size and mean fitness, which could play a role in species where coordinated foraging and nest defence is important (Angulo et al. 2018).

For the halictine bee *L. hemichalceum* the mutualistic nest associations formed by unrelated females appear from the outset to support the GA hypothesis. The paradoxical and unequal distribution of risky activities among unrelated individuals suggests there is some alternative benefit for undertaking risky foraging behaviour. Following the GA model, it is possible that cofounding females actively recruit additional members to undertake foraging activities, which carries the associated risk of mortality. In return, foragers receive direct benefits from increased brood protection, as reported by Kukuk et al. (1998), in which nests where non-forager females were experimentally removed showed a significantly higher brood mortality rate. Comparably, the nesting characteristics of *P. texana* tentatively support the GA prerequisites proposed by Kingma et al. (2014). The narrow foraging window available to *P. texana* suggests that joining a nest opposed to constructing a new one is the optimal strategy. While there is no evidence that foraging tasks are divided among nestmates, it seems that it is favourable to increase group size and effectively enhance protection against predators (Neff and Danforth 1991). Allee effects have also been observed in the bethylid wasp, *Sclerodermus harmandi*. Tang et al. (2014) demonstrated a positive association between host size and the number of foundresses, showing that when multiple foundresses exploited larger hosts, they increased direct fitness benefits in the form of productivity and brood survivability. Allee effects and the GA model are likely to be broadly applicable to species demonstrating group living but are underutilized in explanations precluding kin selection.

Central limit theorem

Other studies have taken a different approach and argued that the benefits of sociality can be explained purely from a statistical theorem. Statistical models such as the central limit theorem (CLT) have been suggested to explain the puzzling correlation between colony size and per capita output (Stevens et al. 2007; Wenzel and Pickering 1991). Wenzel and Pickering (1991) initially showed the applicability of the CLT to social evolution using two species of *Polistes* wasp. They proposed that the CLT provides benefits for sociality, as larger groups are able to better predict food acquisition and adjust brood size accordingly, arguing that while larger groups may have lower mean per capita benefits, the smaller variance of these means is favoured by selection.

Exoneura nigrescens demonstrates a case where increased group membership allows for more stable provisioning of offspring throughout the colony as individuals are able to better match offspring production with floral availability and this in-turn reduces the costs of over producing offspring that cannot be fed (Stevens et al. 2007). Reducing variation in brood loss also extends to enhanced protection against nest invaders. Zammit et al. (2008) showed that social nests of *E. nigrescens* were far more effective at protecting brood loss from ant predation compared to independent nesters. While kin section arguments cannot be ruled out, it seems that sociality in species like *E. nigrescens* has arisen through evolutionary forces that promote selection against variances in fitness rather than the maximisation of fitness (Gillespie 1974).

Reproductive queuing

A key reason for why individuals may remain in a nest in the absence of immediate direct or indirect fitness benefits could be the potential to actively reproduce in the future. The reproductive queuing model is an adapted reproductive skew model that posits subordinates may receive delayed fitness benefits by staying in a nest and inheriting a reproductive role when the dominant dies (Kokko and Johnstone 1999). In primitively eusocial Hymenoptera where nests generally consist of one reproductive dominant and helper subordinates, the reproductive queuing model provides a well-suited framework to explain why subordinates may initially forgo reproduction to help an unrelated queen.

Dominant and subordinate roles are generally widespread and well defined and across many social bee species. Carpenter bees in particular have shown to express these roles in both familial colonies (see Rehan et al. 2010; Stark 1992) and non-relative associations (see Ostwald et al. 2021; Vickruck and Richards 2021). *Ceratina australensis* forms social colonies of either sororal or matrifilial pairings suggesting an avenue for kin selection for any reproductive output generated by the primary nestmate. However, Rehan et al. (2010) found that inclusive fitness for social secondaries did not outweigh the costs of reproducing independently. Instead, secondaries have the opportunity to receive high direct fitness payoffs by remaining in the nest in a reproductive queue. These delayed benefits received by the secondary female create an opening for kin selection to operate, as there is potential for non-reproductive secondary females to solely rely on indirect fitness benefits. Conversely, *X. virginica* falls into the category of unrelated nestmates forming reproductive queues. Vickruck and Richards (2021) posited that individuals will nest with non-relatives to not only improve their position with the reproductive queue and therefore direct fitness, but also to reduce the indirect fitness costs of kin competition.

A plethora of alternative theories have been proposed for the evolution of unrelated co-foundresses in *P. dominula.* However, none have been as widely accepted as the nest-inheritance hypothesis (Leadbeater et al. 2011). While acknowledging that kin selection may play a minor role in explaining cooperative nesting in *P. dominula* via indirect benefits for related subordinates, Leadbeater et al. (2011) found that it is more beneficial for subordinates unrelated to the queen to wait for the chance to inherit the dominant role within the nest compared to reproducing solitarily. Suggesting that future direct fitness benefits for subordinates present a more probable mode of selection for cooperative nesting (Leadbeater et al. 2010). In *R. marginata,* the presence of workers that are predisposed to stay at a nest appears to support kin selection, yet, the behaviour of accepting unrelated nest joiners contradicts it. For this reason, Gadagkar (2016) suggests that alternative evolutionary forces may be at play. Reproductive queuing by unrelated workers suggests there are underlying benefits to staying at a nest opposed to founding a nest solitarily. The reproductive queuing model provides an evolutionary stable strategy for why a dominant *R. marginata* queen may accept nest joiners and why those joiners would abstain reproduction and help increase the direct fitness of the queen (Bang and Gadagkar 2012).

Where kin selection is the widely accepted model for understanding why helpers may forgo reproduction, in the case of the primitively eusocial wasps, *P. dominula* and *R. marginata* and the facultatively social bees, *C. australensis* and *X. virginica* it is evident that social queuing for the potential to obtain direct fitness benefits provides a more realistic framework to explain the evolution of cooperation (Field and Leadbeater 2016; Gadagkar 1990; Gadagkar 2016; Kokko and Johnstone 1999; Leadbeater et al. 2011; Queller et al. 2000; Vickruck and Richards 2021).

Natal Philopatry

Despite the apparent concordance between predictions of kin selection and the biology of thrips with soldier castes, there are reasons to doubt whether kin selection has played a key role in the evolution of soldier castes and whether it is even needed to explain soldier behaviour. (i) Firstly, there are no known thrips species where all soldiers are non-reproductive; instead most female soldiers appear to be reproductive and all male soldiers that have been dissected have large testes (Chapman et al. 2002). (ii) Secondly, in some species females soldiers have ovary sizes that are only marginally smaller than the foundress, and in at least one species females soldiers have larger ovaries than the foundress (Crespi and Mound 1997). (iii) Thirdly, there have been no studies that explicitly evaluated Hamilton’s Rule for gall-forming thrips with soldiers. This raises the question of whether kin selection is needed to explain soldiers in these thrips.

An alternative to kin selection is that ‘soldier’ castes have evolved as a non-dispersing strategy to avoid the high costs of dispersal and instead reproduce in their pre-existing domicile given that it can provide food and shelter resources for more than one generation of thrips. In such a case, selection on potentially non-dispersing individuals would involve a trade-off between the high costs of dispersal and limitations to how many offspring could be produced in a gall that is no longer increasing in size. Dispersal costs are thought to be very high in these minute organisms that have limited fight abilities and the ability to find mates outside of their natal gall, so it is not difficult to imagine that smaller ovary sizes in a female soldier, relative to a foundress, may be a better strategy than risking dispersal. At the same time, the proclivity of soldiers to defend their natal galls from enemies could be due to parental investment and the benefits of protecting their own offspring from enemies.

1.8 Conclusion

Studies of social evolution have generally taken a eusocial perspective. Consequently, this has led to the majority of social evolutionary theories being modelled around highly eusocial species, leaving the more primitive societies ignored. Kin selection presents a highly robust theory to model the social evolution of insects. However, even this seminal theory seemingly falters in scenarios where superficial behaviours appear to support its assumptions. Here we have provided a variety of case studies that appear to fall out of the kin selection paradigm along with others that show how kin selection can operate in conjunction with other forces. These model and non-model species show that helping behaviours and high relatedness do not necessarily preclude kin selection, but suggest an avenue to social nesting that putatively favour the enhancement of direct fitness benefits. To fully understand how sociality evolved in insects requires in-depth studies of the species across the full spectrum of social behaviours. This includes a close examination into what drives early stages in social evolution and whether these could be upscaled to more socially complex species.

1.9 References

Abbot P et al. (2011) Inclusive fitness theory and eusociality. *Nature*. 471:E1–4; author reply E9–10.

Angulo E, Luque GM, Gregory SD, Wenzel JW, Bessa-Gomes C, Berec L, Courchamp F (2018) Allee effects in social species. *Journal of Animal Ecology*. 87:47–58.

Bang A, Gadagkar R (2012) Reproductive queue without overt conflict in the primitively eusocial wasp *Ropalidia marginata*. *Proceedings of the National Academy of Sciences*. 109:14494–14499.

Batra S (1966) Nests and social behavior of halictine bees of India (Hymenoptera: Halictidae). *Indian Journal of Entomology*. 28:375–393.

Boomsma JJ (2009) Lifetime monogamy and the evolution of eusociality. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 364:3191–3207.

Boomsma JJ et al. (2011) Only full-sibling families evolved eusociality. *Nature*. 471:E4–E5.

Boomsma JJ, Gawne R (2018) Superorganismality and caste differentiation as points of no return: how the major evolutionary transitions were lost in translation. *Biological Reviews*. 93:28–54.

Bourke AF (2014) Hamilton's rule and the causes of social evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 369:20130362.

Chapman T, Crespi B, Kranz B, Schwarz M (2000) High relatedness and inbreeding at the origin of eusociality in gall-inducing thrips. *Proceedings of the National Academy of Sciences*. 97:1648–1650.

Chapman TW, Kranz BD, Bejah K-L, Morris DC, Schwarz MP, Crespi BJ (2002) The evolution of soldier reproduction in social thrips. *Behavioral Ecology*. 13:519–525.

Chenoweth LB, Tierney SM, Smith JA, Cooper SJ, Schwarz MP (2007) Social complexity in bees is not sufficient to explain lack of reversions to solitary living over long time scales. *BMC Evolutionary Biology*. 7:246.

Courchamp F, Clutton-Brock T, Grenfell B (1999) Inverse density dependence and the Allee effect. *Trends in Ecology & Evolution*. 14:405–410.

Crespi B (1992*a*) Behavioural ecology of Australian gall thrips (Insecta: Thysanoptera). *Journal of Natural History*. 26:769–809.

Crespi BJ (1992*b*) Eusociality in Australian gall thrips. *Nature*. 359:724.

Crespi B (1994) Three conditions for the evolution of eusociality: are they sufficient? *Insectes Sociaux*. 41:395–400.

Crespi BJ, Mound LA (1997) Ecology and evolution of social behavior among Australian gall thrips and their allies. The evolution of social behavior in insects and arachnids.166–180.

Crespi BJ, Yanega D (1995) The definition of eusociality. *Behavioral Ecology*. 6:109–115.

Crozier RH, Pamilo P (1996) Evolution of social insect colonies. Oxford University Press, Oxford, UK.

Danforth BN, Neff JL, Barretto-Ko P (1996) Nestmate relatedness in a communal bee, *Perdita texana* (Hymenoptera: Andrenidae), based on DNA fingerprinting. *Evolution*. 50:276–284.

Dew RM, Tierney SM, Schwarz MP (2016) Social evolution and casteless societies: needs for new terminology and a new evolutionary focus. *Insectes Sociaux*. 63:5–14.

Evans HE, West-Eberhard MJ (1973) The Wasps. David and Charles.

Field J, Leadbeater E (2016) Cooperation between non-relatives in a primitively eusocial paper wasp, *Polistes dominula*. *Proceedings of the Royal Society B: Biological Sciences*. 371:20150093.

Fletcher JA, Doebeli M (2009) A simple and general explanation for the evolution of altruism. *Proceedings of the Royal Society B: Biological Sciences* 276**:**13–19.

Fletcher JA, Zwick M, Doebeli M, Wilson DS (2006) What's wrong with inclusive fitness? *Trends in Ecology & Evolution*. 21**:**597.

Foster KR, Wenseleers T, Ratnieks FL (2006) Kin selection is the key to altruism. *Trends in Ecology & Evolution*. 21:57–60.

Gadagkar R (1990) Origin and evolution of eusociality: a perspective from studying primitively eusocial wasps. *Journal of Genetics*. 69:113–125.

Gadagkar R (2016) Evolution of social behaviour in the primitively eusocial wasp *Ropalidia marginata*: do we need to look beyond kin selection? *Philosophical Transactions of the Royal Society B: Biological Sciences*. 371:20150094.

Gardner A, Grafen A (2009) Capturing the superorganism: a formal theory of group adaptation. *Journal of Evolutionary Biology*. 22:659–671.

Gardner A, West SA, Wild G (2011) The genetical theory of kin selection. *Journal of Evolutionary Biology*. 24:1020–1043.

Gibbs J, Brady SG, Kanda K, Danforth BN (2012) Phylogeny of halictine bees supports a shared origin of eusociality for *Halictus and Lasioglossum* (Apoidea: Anthophila: Halictidae). *Molecular Phylogenetics and Evolution*. 65:926–939.

Gillespie JH (1974) Natural selection for within-generation variance in offspring number. *Genetics*. 76:601–606.

Hamilton WD (1964*a*) The genetical evolution of social behvaiour I. *Journal of Theoretical Biology*. 7:1–16.

Hamilton WD (1964*b*) The genetical evolution of social behvaiour II. *Journal of Theoretical Biology*. 7:17–52.

Herre EA, Wcislo WT (2011) In defence of inclusive fitness theory. *Nature*. 471:E8–E9.

Hughes WOH, Oldroyd BP, Beekman M, Ratnieks FLW (2008) Ancestral monogamy shows kin selection is key to the evolution of eusociality. *Science*. 320:1213–1216.

Kingma SA, Santema P, Taborsky M, Komdeur J (2014) Group augmentation and the evolution of cooperation. *Trends in Ecology & Evolution*. 29:476–484.

Kocher SD, Paxton RJ (2014) Comparative methods offer powerful insights into social evolution in bees. *Apidologie*. 45:289–305.

Kokko H, Johnstone RA (1999) Social queuing in animal societies: a dynamic model of reproductive skew. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 266:571–578.

Kokko H, Johnstone RA (2001) The evolution of cooperative breeding through group augmentation. *Proceedings of the Royal Society B: Biological Sciences*. *268*:187–196.

Kukuk PF, Bitney C, Forbes SH (2005) Maintaining low intragroup relatedness: evolutionary stability of nonkin social groups. *Animal Behaviour*. 70:1305–1311.

Kukuk PF, Sage GK (1994) Reproductivity and relatedness in a communal halictine bee *Lasioglossum* (*Chilalictus*) *hemichalceum*. *Insectes Sociaux*. 41:443–455.

Kukuk PF, Ward SA, Jozwiak A (1998) Mutualistic benefits generate an unequal distribution of risky activities among unrelated group members. *Naturwissenschaften*. 85:445–449.

Leadbeater E, Carruthers JM, Green JP, Rosser NS, Field J (2011) Nest inheritance is the missing source of direct fitness in a primitively eusocial insect. *Science*. 333:874–876.

Leadbeater E, Carruthers JM, Green JP, Van Heusden J, Field J (2010) Unrelated helpers in a primitively eusocial wasp: is helping tailored towards direct fitness? *PLoS One*. 5:e11997.

Liao X, Rong S, Queller DC (2015) Relatedness, conflict, and the evolution of eusociality. *PLoS Biology*. 13:e1002098.

Liebert AE, Starks PT (2006) Taming of the skew: transactional models fail to predict reproductive partitioning in the paper wasp *Polistes dominulus*. *Animal Behaviour*. 71:913–923.

Lin N, Michener CD (1972) Evolution of Sociality in Insects. *The Quarterly Review of Biology*. 47:131–159.

Linksvayer TA, Johnson BR (2019) Re-thinking the social ladder approach for elucidating the evolution and molecular basis of insect societies. *Current Opinion in Insect Science*. 34:123–129.

Michener CD (1969) Comparative social behavior of bees. *Annual Review of Entomology*. 14:299–342.

Neff JL, Danforth BN (1991) The nesting and foraging behavior of *Perdita texana* (Cresson)(Hymenoptera: Andrenidae). *Journal of the Kansas Entomological Society*. 64:394–405.

Nonacs P, Reeve HK (1995) The ecology of cooperation in wasps: causes and consequences of alternative reproductive decisions. *Ecology*. 76**:**953–967.

Nowak MA, Tarnita CE, Wilson EO (2010) The evolution of eusociality. *Nature*. 466:1057–1062.

O'Donnell S, Jeanne RL (1992) Lifelong patterns of forager behaviour in a tropical swarm-founding wasp: effects of specialization and activity level on longevity. *Animal Behaviour*. 44:1021–1027.

Ohkubo Y, Yamamoto T, Ogusu N, Watanabe S, Murakami Y, Yagi N, Hasegawa E (2018) The benefits of grouping as a main driver of social evolution in a halictine bee. *Science Advances*. 4:e1700741.

Ostwald MM, Dahan RA, Shaffer Z, Fewell JH (2021) Fluid Nest Membership Drives Variable Relatedness in Groups of a Facultatively Social Bee. *Frontiers in Ecology and Evolution*. 9:767380.

Perry S, Chapman T, Schwarz M, Crespi B (2004) Proclivity and effectiveness in gall defence by soldiers in five species of gall-inducing thrips: benefits of morphological caste dimorphism in two species (*Kladothrips intermedius* and *K. habrus*). *Behavioral Ecology and Sociobiology*. 56:602–610.

Queller DC, Strassmann JE (1998) Kin selection and social insects. *Bioscience*. 48:165–175.

Queller DC, Zacchi F, Cervo R, Turillazzi S, Henshaw MT, Santorelli L, Strassmann JE (2000) Unrelated helpers in a social insect. *Nature*. 405:784–787.

Reeve HK (2001) In search of unified theories in sociobiology: help from social wasps. Model systems in behavioral ecology. Princeton University Press, Princeton, NJ. 57–71

Rehan SM, Richards MH, Schwarz MP (2010) Social polymorphism in the Australian small carpenter bee, *Ceratina (Neoceratina) australensis*. *Insectes Sociaux*. 57:403–412.

Rehan SM, Richards MH, Adams M, Schwarz MP (2014) The costs and benefits of sociality in a facultatively social bee. *Animal Behaviour*. 97:77–85.

Richards MH (2019) Socially polymorphic bees as model organisms for studying the evolution of eusociality. *Insectes Sociaux*. 66:3–4.

Schwarz MP (1987) Intra-colony relatedness and sociality in the allodapine bee *Exoneura bicolor*. *Behavioral Ecology and Sociobiology*. 21:387–392.

Shakarad M, Gadagkar R (1997) Do social wasps choose nesting strategies based on their brood rearing abilities? *Naturwissenschaften*. 84:79–82.

Silberbauer LX, Schwarz MP (1995) Life cycle and social behaviour in a heathland population of the allodapine bee, *Exoneura bicolor*. *Insectes Sociaux*. 42:201–218.

Spessa A, Schwarz MP, Adams M (2000) Sociality in *Amphylaeus morosus*. *Entomological Society of America*. 93:684–692.

Stark RE (1992) Cooperative nesting in the multivoltine large carpenter bee *Xylocopa sulcatipes* Maa (Apoidea: Anthophoridae): do helpers gain or lose to solitary females? *Ethology*. 91:301–310.

Stevens MI, Hogendoorn K, Schwarz MP (2007) Evolution of sociality by natural selection on variances in reproductive fitness: evidence from a social bee. *BMC Evolutionary Biology*. 7:153.

Stow A, Silberbauer L, Beattie AJ, Briscoe DA (2007) Fine-Scale Genetic Structure and Fire-Created Habitat Patchiness in the Australian Allodapine Bee, *Exoneura nigrescens* (Hymenoptera: Apidae). *Journal of Heredity*. 98: 60–66.

Strassmann JE, Page RE, Robinson GE, Seeley TD (2011) Kin selection and eusociality. *Nature*. 471:E5–E6.

Tang X, Meng L, Kapranas A, Xu F, Hardy IC, Li B (2014) Mutually beneficial host exploitation and ultra-biased sex ratios in quasisocial parasitoids. *Nature Communications*. 5:1–7.

Vickruck JL, Richards MH (2018) Linear dominance hierarchies and conditional reproductive strategies in a facultatively social carpenter bee. *Insectes Sociaux*. 64:619–629.

Vickruck JL, Richards MH (2021) Competition Drives Group Formation and Reduces Within Nest Relatedness in a Facultatively Social Carpenter Bee. *Frontiers in Ecology and Evolution.* 9:738809.

Wcislo W, Tierney S (2009) Evolution of communal behavior in bees and wasps: an alternative to eusociality. Organization of insect societies from genome to sociocomplexity. Harvard University Press, Massachusetts. 148–169.

Wenzel JW, Pickering J (1991) Cooperative foraging, productivity, and the central limit theorem. *Proceedings of the National Academy of Sciences*. 88:3–38.

Wheeler W (1928) The Social Insects: Their Origin and Evolution, Kegan Paul, Trench, Trubner and Co Ltd London, xviii.

Wilson EO (1971) The insect societies. The insect societies. Belknap Press of Harvard University Press.

Wilson EO (2008) One giant leap: how insects achieved altruism and colonial life. *BioScience*. 58:17–25.

Wilson EO, Hölldobler B (2005) Eusociality: origin and consequences. *Proceedings of the National Academy of Sciences of the United States of America*. 102:13367–13371.

Yagi N, Hasegawa E (2012) A halictid bee with sympatric solitary and eusocial nests offers evidence for Hamilton's rule. *Nature Communications*. 3:939.

Zammit J, Hogendoorn K, Schwarz MP (2008) Strong constraints to independent nesting in a facultatively social bee: quantifying the effects of enemies-at-the-nest. *Insectes Sociaux*. 55:74–78.

Glossary of terms

Kin selection: A foundational theory of modern social behaviour that explains how altruistic behaviour can evolve and be maintained. Individuals may sacrifice reproduction to aid the reproductive efforts of their kin. Hamilton (1960a,b) formulated this principle into the equation $r.b>c$, which states that altruistic alleles may be selected for when the benefit of helping (b) multiplied by the relatedness between the recipient of help and the altruist (r) is greater than the cost of sacrificing individual reproduction through reproducing alone (c).

Group augmentation: Individuals actively or passively recruit additional members to increase the overall group size. Larger group sizes are thought to increase survivability and reproduction through similar principals associated with cooperative breeding where helping behaviour is selected for via a mutual benefit to actor and recipient. The chances of former helpers receiving delayed fitness benefits from other group members increases as group size increases and the number of positive interactions increases.

Allee effects: Theorised by W.C. Allee, the Allee effect is a concept that states there is a positive correlation between population size and components of an individual's fitness. Allee effects can be recognised as a density-dependent phenomenon that occurs when individuals gain increasing benefits from positive interactions in an increasing population size. In social evolution, this idea relates to how social interactions, such as resource sharing, helping behaviours and increased defence can enhance per capita gains and influence population dynamics.

Reproductive queuing: An adapted reproductive skew model that accounts for the delayed fitness benefits of remaining in a group as a subordinate for the potential to take over as the dominant breeder in the future. Individuals in hierarchal societies remain in the group instead of dispersing to nest alone for the potential to inherit the dominant breeder status after the death of the current dominant.

Central limit theorem: Statistical principal that states that as the sample size in a population increases, variance in the sample mean decreases. In an ecological and evolutionary context, this principle provides a purely statistical explanation for why larger group sizes are maintained despite lower per capita reproductive output. These principles are derived from the same arguments proposed by Gillespie (1974), who stated selection should act against variance in fitness.

Table 1. Classification of social terminology developed for insect societies. Adapted from Crespi and Yanega (1995), Dew et al. (2016) and, Michener (1969).

		Reproductive division of labour	**Overlapping generations**	**Parental care**	**Morphological castes**
Solitary		–	–	–	–
Subsocial		–	–	+	–
Parasocial	Casteless	–	–	–	–
	Communal	–	–	–	±
	Semisocial	±	–	+	+
Eusocial	Primitively	+	+	+	–
	Advanced	+	+	+	+

Table 2. Social characteristics and alternative selection modes proposed for case studies.

Taxon/species	Social classification	Intracolony relatedness	Explanation for intracolony relatedness	Applicability of kin selection	Alternative hypothesis	Alternative model	Reference
Bees							
Ceratina australensis	Subsocial	**High** $r \approx 0.79$	Two full-sisters remain in their natal nest	✓✘	Nest inheritance, direct fitness benefits	Natal philopatry, Reproductive queuing	Rehan *et al.* (2014)
Lasioglossum hemichalceum	Communal	**Low** $r \approx 0.13$	Dispersal from natal nest to initiate new colonies with unrelated females	✘	Mutualistic association	Allee effects, Group augmentation	Kukuk & Sage (1994)
Perdita texana	Communal	**Low** $r \approx 0.0425$	Frequent co-founding of new nests	✘	Mutualistic association, Assured fitness returns	Allee effects, Group augmentation	Danforth *et al.* (1996)
Xylocopa virginica	Semisocial	**Low** $r \approx 0.21$	Dispersal from natal nest to avoid kin competition	✘	Nest inheritance, Assured fitness returns	Reproductive queuing	Vickruck & Richards (2018; 2021)
Exoneura nigrescens	Primitively eusocial	**Moderate** $r \approx 0.478$	Serial polyandry	✓✘	Selection against variances	Central limit theorem	Schwarz (1987), Stevens *et al.* (2007), Stow *et al.* (2007)
Wasps							
Polistes dominula	Primitively eusocial	**Low/moderate** $r = 0.13\text{-}0.53$	Frequent co-founding of new nests, subordinate reproduction	✓✘	Nest inheritance	Reproductive queuing, Group augmentation	Field & Leadbeater (2015), Leadbeater *et al.* (2010)
Ropalidia marginata	Primitively eusocial	**Low/moderate** $r = 0.22\text{-}0.46$	Serial polyandry	✓✘	Assured fitness returns	Reproductive queuing	Gadagkar (2016)
Gall-forming thrips							
	Eusocial	**High** $r = 0.64\text{-}0.92$	High levels of inbreeding	✓✘	Direct fitness benefits	Natal philopatry	Crespi (1992), Chapman *et al.* (2002)

Chapter 2 - Description and novel host records for a new species of Australian mutillid wasp (Hymenoptera: Mutillidae) from hylaeine bee nests (Hymenoptera: Colletidae)

This chapter has been published in Austral Entomology

https://doi.org/10.1111/aen.12414

Lucas R. Hearn[1*], Kevin A. Williams[2], Mark I. Stevens[3, 4], Michael P. Schwarz[1], Olivia K. Davies[1] and Ben A. Parslow[1]

[1]*College of Science and Engineering, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia.*

[2]*California State Collection of Arthropods, California Department of Food & Agriculture, 3294 Meadowview Road, Sacramento, CA 95832-1448, U.S.A.*

[3]*Earth and Biological Sciences, South Australian Museum, GPO Box 234, Adelaide, SA 5001, Australia.*

[4]*School of Biological Sciences, University of Adelaide, SA 5005, Australia.*

Corresponding author: Lucashearn7@gmail.com*

2.1 Abstract

A new mutillid wasp, *Ephutomorpha tyla* Hearn, Williams & Parslow sp. nov., is described from adult female and male specimens from the Dandenong Ranges in Victoria, Australia. Adult mutillids were repeatedly found in nests of the hylaeine bee *Amphylaeus morosus* (Smith, 1879) (Hymenoptera: Colletidae: Hylaeinae) and reared from host nest cells. This represents the first recorded host-parasite association between Mutillidae and hylaeine bee species.

Key words: Hylaeinae, parasitoids, host-parasite association, velvet ant.

2.2 Introduction

Mutillid wasps, commonly known as velvet ants, comprise a diverse family of solitary wasps, with around 4300 described species, occurring on all continents except Antarctica (Luz et al. 2016; Brothers and Lelej 2017). These wasps are broadly characterised by their extreme sexual dimorphism, wherein females are apterous and males are fully winged. These extreme morphological differences make it difficult to associate sexes without biological or molecular data (Brothers 2018) and contribute to the many taxonomic issues that plague this family.

The systematics of Australian mutillids are poorly understood and only two of the eight known subfamilies have been reported from Australia (Brothers and Lelej 2017). Most of the Australasian fauna have been ascribed to a single genus, *Ephutomorpha* André, 1902, which is likely a portmanteau taxon (Brothers and Finnamore 1993). Recent description of the genus *Aglaotilla* Brothers, 2018 has prompted further discussion regarding the state of the Australasian taxa, which currently places nearly 250 described species into ten genera (Brothers 2018). Additionally, host associations for mutillid wasps are largely unknown and present a marked discrepancy in the already under-studied biology of this family.

Most mutillids are parasitoids of solitary hosts but estimates of recorded mutillid natural history suggest that only 2-3% of these associations are known (Brothers 1989). Only one species in the bee family Colletidae has been associated with a mutillid parasite in Australia. Rayment (1953) found the mutillid wasp, *Ephutomorpha auricrucis,* was parasitic on the larvae of *Xanthesma maculata* (Smith, 1879) (as *Euryglossa maculata*), however, to date, there are no records of a host-parasite relationship between any Mutillidae and Hylaeinae species (Taylor et al. 2019).

Here we describe a new species, *Ephutomorpha tyla* sp. nov. Hearn, Williams & Parslow, and present a new host record for the genus *Ephutomorpha* André, the hylaeine bee *Amphylaeus morosus* (Smith, 1879) (Apidae: Colletidae: Hylaeinae).

2.3 Materials and Methods

Fifteen specimens of *Ephutomorpha tyla* were collected in nests of *Amphylaeus morosus* (identification followed Houston 1975) from the Gembrook and Cockatoo regions of the Dandenong Ranges, Victoria between the 4th and 6th of December 2017. Adult female *E. tyla* were removed from the inner entrances of each nest and placed in 99% ethanol for preservation. Nests were opened in late December 2017 and early January 2018 and their contents carefully examined. Parasitised brood cells were reared at room temperature until eclosion.

Specimens were examined at the South Australian Museum (SAMA) and imaged using a Nikon SMZ1500. Images were taken using a Visionary Digital BK+ imaging system with a combination of Canon EOS 7D camera, and a 5D Mark II (Australian Centre for Evolutionary Biology and Biodiversity at the University of Adelaide). Images were produced using Zerene Stacker, Zerene Systems LLC, software and cropped and resized in Photoshop CS5. Male genitalia were imaged in 70% ethanol.

We follow the latest higher classification of Mutillidae presented by Brothers and Lelej (2017) that places *Ephutomorpha* in the tribe Dasymutillini. Terminology and measurements for adult body morphology follow Bartholomay et al. (2018) and the Hymenoptera Anatomy Consortium (2018). Terminology for surface sculpturing follows Harris (1979). We use the abbreviations F, S and T for antennal flagellomeres, metasomal sterna and terga respectively; these are followed by a number to denote the relevant segment (e.g. T2, T3, etc., second, third, etc. terga). We use the term “simple setae” for all setae with smooth surfaces that do not have barbs, and “brachyplumose setae” for setae with barbs less than or equal to the setal diameter. Morphometric measurements are presented as the mean followed by the range. The head width was determined as the widest possible measurement in dorsal view; the pronotal width was measured between the outside edges of the pronotal spiracles in dorsal view. For ease of comparison and to facilitate identification without dissecting the genitalic capsule, the cuspis, digitus and paramere measurements are taken in dorsal view from the apical margin of the parapenial lobe to the apex of each respective structure. Using this method, all measurements can be taken from the dorsal view and a single anchor point can be used for each measurement. These are not actual measurements of structure length, but an index to compare relative lengths; length ratios for a given genitalic structure are compared against the free paramere length (FPL), the distance from the apex of the parapenial lobe to the apex of the paramere.
Type and examined material are deposited in the following collections: SAMA = South Australian Museum, Adelaide, SA, Australia; ANIC = Australian National Insect Collection, Canberra, ACT, Australia; NMV = Museums Victoria, Melbourne, VIC, Australia.

2.4 Taxonomy

Ephutomorpha André, 1902

Type species. *Mutilla aurata* Fabricius, 1775 by original designation.

The genus *Ephutomorpha* has been treated as a dumping ground for Australian Sphaeropthalminae and, therefore, cannot be consistently defined by any combination of characters. Rather, the members of this genus are recognized only by their circular to subcircular eyes (a defining feature of the subfamily Sphaeropthalminae) and by the absence of traits used to define the few other described Australian genera (see Ashmead 1899; Brothers 1971, 1994, 2012, 2018; Lelej 1983).

Ephutomorpha tyla Hearn, Williams & Parslow sp. nov.

(Figs 1–3)

http://zoobank.org/urn:lsid:zoobank.org:act:9E3947D5-D62B-41A0-BE97-C912AADACE63

Material examined

Holotype

♀, AUSTRALIA, Victoria, Gembrook, Dandenong Ranges, 4–6.xii.2017, M.P. Schwarz, ex nests of *Amphylaeus morosus* (**SAMA: 32-036209)**.

Specimen damage. Left fore leg removed for DNA sequencing.

Paratypes

15 ♀♀ & 1♂: AUSTRALIA, Victoria, Gembrook and Cockatoo, Dandenong Ranges, 4–6.xii.2017, M.P. Schwarz, ex nests of *Amphylaeus morosus*, 1♀ pinned (**SAMA: 32-036210), 6**♀ preserved in 98% ethanol (**SAMA: 32-036211 – SAMA: 32-036216), 1**♂ pinned (**SAMA: 32-036217**), 4♀ preserved in 98% ethanol (**ANIC: 32-141534 – 32-141537**), 4♀ preserved in 98% ethanol (**NVM: T-22397 – T-22400**).

Other material

1♀**,** AUSTRALIA, New South Wales, Hazelbrook, Blue Mountains, 27.vii.2017, J.B. Dorey, ex nests of *Amphylaeus morosus* (**SAMA: 32-036225)**.

Diagnosis

FEMALE. This species is recognized by the following unique combination of characters, presented here in the order of their usefulness for narrowing down the immense diversity of *Ephutomorpha s.l.*: T6 lacks a defined pygidial plate; T1 is broadly sessile with T2; the mesosoma has the dorsal and lateral faces separated by an incomplete carina or angle; S2 is armed with a medial swollen protuberance; the head and mesosoma are dark metallic blue, contrasting with orange legs and predominantly black metasoma; T2-5 each have a broad apicomedial patch of dense pale yellow setae; and T2-3 have the cuticle pale yellow beneath this setal mark. MALE. Likewise, the male is recognized by a unique combination of characters, here presented in a strategic order: the axilla is unarmed; the mandible lacks a ventral tooth basally; T1 is narrowly sessile with T2; S2 has a swollen medial protuberance; the body is mostly black except for the orange legs and dark orange T1; T3-7 are clothed mostly (T3-5 entirely) with pale yellow setae. The genitalic features of this insect are also unique, especially in having the paramere sinuous and tapering, the parapenial lobe with a short incurved truncate tooth, the cuspis short, the paracuspis well-defined, and the penis valve extending beyond the parapenial lobe.

Description

FEMALE. Body length 10.9 (10.3 – 11.3) mm. *Colouration*. Head and mandible metallic blue, antenna mainly black with base of scape dark brown, propleuron metallic blue, mesosoma metallic blue except for ventral posterior corner of mesepisternum and posterior corner of metapleuron orange. Coxae orange, trochanters orange, femora orange, tibiae dark orange, tarsi dark orange, tarsi 5 and tarsal claws dark brown, tibial spurs cream, T1 light brown with black spot dorso-medially and with apical pale integumental band, T2–6 black with light-brown patch dorso-medially on posterior of T2 and T3, S1–5 black. Head with scattered sub-appressed simple white setae, interspersed with erect simple black setae around dorsal eye margin, erect simple white setae on vertex and ventral eye margin, dense erect simple setae on clypeal margin, pedicel and scape with sub-appressed simple white setae. Mesosoma with sub-appressed simple white setae, interspersed with erect and sub-appressed simple black setae dorsally, short simple white erect setae on anterior of mesoscutum, long erect simple white setae on propodeum, loose patch of sub-appressed simple whitish setae anterolaterally on mesonotum, legs with simple white erect setae. Metasoma with T1, T2–6 laterally, and S1–5 with sparse erect simple white setae; T2 disc, T2 apicolaterally, T3–5 sublaterally, and T6 with sub-appressed simple black setae interspersed with erect simple black setae, lateral felt line on T2 black with sub-appressed short simple white setae; T1 apical band and T2–5 apicomedial patch with denser sub-appressed simple pale-yellow setae. *Head*. Head width 1.04 (1.01 – 1.09) × pronotal width. Head areolate, clypeus without distinct teeth or ridges, antennal scrobe with dorsal carina, F1 length 1.95 (1.66 – 2.14) × pedicel length, F2 length 1.44 (1.25 – 1.57) × pedicel length. *Mesosoma*. Mesosomal length 1.17 (1.16 – 1.18) × width. Humeral carina scarcely defined. Mesosomal dorsum areolate, intervals clearly defined, areolations slightly tighter anteriorly than posteriorly. Lateral pronotal face imbricate dorsally, transverse rugose-striate ventrally; mesopleuron smooth dorso-anteriorly, areolate-rugose posteriorly; metapleuron smooth anteriorly, rugose-imbricate posteroventrally; lateral propodeal face imbricate. Metapleural-propodeal suture carinate to posteroventral margin of endophragmal pit. Dorsal and lateral propodeal faces separated by short sinuate carina, dorsal and posterior propodeal faces evenly rounded together. *Metasoma*. T1 punctate, shape evenly rounded, sessile with T2; T2 punctate, slightly wider than long (width 1.08 (1.07 – 1.09) × length), felt line 0.2 × T2 length; T3–5 punctate; T6 punctate, lacking pygidial plate. S2 punctate, punctures sparser and coarser than T2, with longitudinal swollen protuberance in basal half; S3–5 punctate.

MALE. Body length 12.8 mm. *Colouration*. Head and mandible black, antenna largely light brown with shades of black and scape light brown, propleura black, mesosoma black except for ventral posterior corner of mesepisternum and posterior corner of metapleuron orange. Legs light orange, tarsi 5 and tarsal claws dark brown, tibial spurs on fore legs light brown, mid and hind legs dark brown, T1 dark orange, T2–7 black. Wings with light infuscation on apical half becoming light brown near apex on fore wing and apically on hind wing, veins brown. Head with sub-appressed simple white setae, interspersed with erect simple black setae around dorsal eye margin and some scattered erect brachyplumose black setae on posterior margin of vertex, erect simple white setae on vertex and ventral eye margin, dense erect simple white setae on clypeal margin, pedicel and scape with erect simple white setae. Pronotum with sub-appressed simple white setae, interspersed with erect simple black setae. Mesoscutum with sub-appressed simple white setae, interspersed with erect simple black setae, some scattered brachyplumose black setae anteriorly and short erect simple white setae on anterior, mesopleuron with erect simple white setae ventrally, dense short simple white setae surrounding pronotal spiracle, metapleuron with dense sub-appressed simple white setae, long simple white erect setae on propodeum. Legs with simple white erect setae, interspersed with long simple black setae. Fore wing with simple short brown/black setae. Metasoma with loose patch of erect simple white setae on T1, T2-6 laterally, and S1-5 with sparse erect simple white setae; T2 apicomedially with sub-appressed simple black setae and apicolaterally with erect simple black setae; T3-5 sublaterally, and T6 with sub-appressed simple black setae interspersed with erect simple black setae, felt line black with short sub-appressed simple white setae; T2 with small basomedial whitish setal patch, T3-6 apicomedial band with denser sub-appressed simple pale yellow setae. *Head*. Head width 0.93 × pronotal width. Head areolate, clypeus without distinct teeth or ridges, mandible tridentate apically, unarmed ventrally. Ocelli small; ocellocular distance 3.0 × length of lateral ocellus, interocellar distance subequal to lateral ocellar length. F1 length 2.14 × pedicel length, F2 length 1.57 × pedicel length. *Mesosoma*. Mesosomal length 1.49 × width. Pronotum and mesoscutum rugulose-lacunose; notaulus complete, distinct; parapsidal line nearly reaching anterior margin of mesoscutum, distinct; tegula broadly convex, smooth with margins sparsely punctate, mesoscutellum rugulose-lacunose; dorsal propodeal face areolate. Mesopleuron rugulose-lacunose, metapleuron mostly smooth, lateral propodeal face areolate to glabrous antero-ventrally. Fore wing with veins encompassing basal 0.85 × total wing length; with three submarginal cells, third submarginal cell apical bounding vein faint; marginal cell acute apically, its length along costal margin 2.0 × stigma length. *Metasoma*. T1 narrowly sessile with T2, T1 puncticulate, T2 slightly wider than long (width 1.36 × length), felt line 0.33 × T2 length. T3-6 punctate, T7 convex, sparsely punctate with interspaces smooth, S2 punctate with medial swollen protuberance, punctures sparser and coarser than T2, S3-5 puncticulate, S6 puncticulate. Hypogidium punctate, bidentate posteriorly. *Genitalia.* Paramere sinuous in dorsal view, laterally compressed broad basal portion shallowly downcurving and tapering to slightly upturned acute apex in lateral view; with short setal brush in basal half and few long-incurved setae at apex. Parapenial lobe apico-dorsally with incurved truncate tooth-like lobe. Cuspis extending 0.34 × FPL, laterally compressed, tapering to rounded apex with dense thickened setae along inner face; paracuspis laterally-compressed, tapering to subacute apex, incurving, with scattered setae, extending 0.20 × FPL. Digitus narrow, laterally compressed, incurved in dorsal view, asetose, extending 0.23 × FPL. Penis valve extending 0.15 × FPL, baso-dorsally expanded, ventrally tridentate, apical teeth approximate, subequal in size, basal tooth large, antero-ventrally curved.

Distribution

Known only from the Dandenong Ranges, Victoria, Australia and a single specimen collected from Hazelbrook, New South Wales, Australia. These regions consist of natural temperate montane forest, dominated by tall Mountain Ash or Blue Gum forest with a ferny undergrowth.

Etymology

Named after Tyla Mary Cousins, partner of LRH in recognition of her support for LRH’s continuing research work. Treat as noun in apposition.

Remarks

Asymmetries are present in the legs of the male paratype likely due to malformation during pre-eclosion. Right hind leg tibia malformed (undeveloped, wafer-like cuticle) with tarsus missing, right fore leg tibia and tarsus pale in colour. Pollen grains on some female specimens.
In the female having a largely metallic blue body and T6 without a defined pygidial plate, this species is superficially similar to the type species of *Ephutomorpha*, *E. aurata* (Fabricius, 1775). This female's broadly sessile T1 and different mesosomal shape (wherein the dorsal and lateral faces are separated by an angle or carina) are unlike *E. aurata*, though. The combination of these two features does, however, allow *E. tyla* to be superficially associated with various forms, including *E. varipes* (André, 1895), up to 13 other named females, and 25 apparently undescribed morphospecies (KAW pers. obs.). Within this group, *E. tyla* is apparently most similar to *E. aeneiventris* (André, 1898) from Queensland, in having T1 with a pale yellow-orange sub-apical band and S2 with a swollen protuberance.

At this point, subtle variations in mesosomal and T1 shape cast doubt on which species belong to this group and whether the group itself is sufficiently different from *E. aurata* to warrant establishment of a new genus. Male morphology could help define the genus limits, but neither *E. aurata* nor any of the 14 described females that potentially belong to this group are known from both sexes. In having the metasoma mostly clothed with pale yellow setae, the male of *E. tyla,* is similar to *E. aurovestita aurovestita* (André, 1895) and its subspecies, *E. a. fulvipes* André, 1903 and *E. a. pallidinervis* André, 1903. It can be immediately separated from the nominotypical subspecies by having orange legs (legs black in *E. a. aurovestita*) and from the other two subspecies by having the tegulae and antennae blackish (tegulae and antennae orange and concolourous with legs in *E. a. fulvipes* and *E. a. pallidinervis*). Additionally, the genitalia of *E. tyla* differ from examined specimens of *E. aureovestita* in their shortened cuspis and sinuous paramere (KAW pers. obs.).

2.5 Biology

Ephutomorpha tyla was frequently observed in nests of the hylaeine bee *Amphylaeus morosus* (Fig. 4), a facultatively social and casteless bee with a distribution throughout south-eastern Australia, westwards to the Portland region in Victoria and northwards to Tin Can Bay in southern Queensland. These habitats span coastal/inland heath in the northern- and western- most distributions, to temperate montane forests in the mid and southern regions of the *A. morosus* range. Mutillid parasitoids have not been collected in nests in heathland habitats where *A. morosus* form nests in dead *Xanthorrhoea* spp. flower scapes and seem to be biogeographically restricted to the central montane regions where *A. morosus* nest more densely in the fallen fronds of *Cyathea australis.* Nests are founded solitarily but may be joined by one or more females to cooperatively rear brood throughout spring and early summer (Spessa et al. 2000). Cooperative nesting may entail nest guards to protect the nest entrance, which could make it more difficult for parasitoids such as *E. tyla* to infiltrate the nest, as has been reported in many social halictines (Brothers et al. 2000).

Nests dissected in late December 2017 and early January 2018 were carefully examined once *A. morosus* brood had reached adult eclosion. Fourteen nests contained a single adult female *E. tyla*, however, only seven of these nests contained parasitised brood. This could suggest that this mutillid species may wait until host brood have reached a late stage of development before parasitising them, which is consistent with the life-histories of numerous other mutillid species (Brothers 1972, 1989). An additional two nests contained parasitised brood but no adult female *E. tyla* at the time of collection, suggesting that this mutillid may move between different host nests or that they can be ejected by hosts after egg deposition. The parasitised nests contained up to 10 sealed bee brood cells, with 66.7% (34/51) of the total brood cells parasitised by *E. tyla*, while the percentage of parasitised cells per nest ranged from 25% to 100%. The parasitised cells contained white cocoons, which occupied the entirety of the *A. morosus* brood cell, similar to those described in Brothers *et al.* (2000), suggesting that only one parasitoid egg is deposited in each host brood cell. Additionally, 8.7% (34/393) of the total population brood cells were occupied by *E. tyla*, indicating that *E. tyla* is a parasitoid of *A. morosus* brood.

This association of the newly described mutillid species, *E. tyla*, with the hylaeine bee, *A. morosus*, represents the first record of a mutillid parasite for this bee subfamily. Although isolated species descriptions are far from ideal and the genus placement of this species is tenuous, it is important to provide a formal name for this new species to allow novel host associations to be further explored. When both sexes are considered, the putative new genus that will house this species includes up to 20 named forms with their types housed in various collections and nearly 40 additional undescribed forms (KAW, pers. obs.). The photographs, descriptions, and biological information provided here, however, can be a useful starting point for future studies in that apparent genus, especially considering that *E. tyla* is the only species in this group currently recognized from both sexes.

2.6 Acknowledgements

We thank Denis Brothers and Arkady Lelej for their helpful and thorough reviews of this manuscript. KAW also thanks Denis Brothers for his insights and advice about the Australian mutillid fauna. The fieldwork was funded by a Holsworth Wildlife Research Endowment grant awarded to OKD and in part funded by an ABRS National Taxonomy Research Grant Programme grant RF217-14 to BAP.

2.7 References

André E (1895) Mutillides d'Australie nouvelles ou imparfaitement connues. *Mémoires de la Société Zoologique de France*. 8:475–517.

André E (1898) Contribution a la connaissance des Mutillides de l'Australie. *Mémoires de la Société Zoologique de France*. 11:256–308.

André E (1903) Quatrième contribution a la connaissance des Mutillides de l'Australie. *Mémoires de la Société Zoologique de France*. 15:240–278.

Ashmead WH. 1899. Superfamilies in the Hymenoptera and generic synopses of the families Thynnidae, Myrmosidae, and Mutillidae. *Journal of the New York Entomological Society* 7:45–60.

Bartholomay PR, Williams KA, Lus DR, Oliveira ML (2018) New species of *Traumatomutilla* André in the *T. tabapua* and *T. integella* species-groups (Hymenoptera, Mutillidae). *Zootaxa*. 4433:361–385.

Brothers DJ (1971) *Ascetotilla*, a new genus of Mutillidae from New Guinea (Hymenoptera). *Pacific Insects*. 13:471–485.

Brothers DJ (1972. Biology and immature stages of *Pseudomethoca f. frigida*, with notes on other species (Hymenoptera: Mutillidae). *The University of Kansas Science Bulletin* 50:1–38.

Brothers DJ (1989) Alternative life-history styles of mutillid wasps (Insecta, Hymenoptera). In: *Alternative life-history styles of animals* (M.N. Bruton, Ed.) pp. 279-291. Kluwer Academic Publishers, Dordrecht.

Brothers DJ (1994) A new genus and four new species of Mutillidae associated with *Brachyponera lutea* Mayr (Formicidae) in Western Australia (Hymenoptera). *Journal of the Australian Entomological Society*. 33:143–152.

Brothers DJ (2012) The new genus *Ancistrotilla*, with new species from Vanuatu and New Caledonia (Hymenoptera: Mutillidae). *Zoosystema*. 34:223–251.

Brothers DJ (2018) *Aglaotilla*, a new genus of Australian Mutillidae (Hymenoptera) with metallic coloration. *Zootaxa*. 4415:357–368.

Brothers DJ, Finnamore AT (1993) Superfamily Vespoidea. in *Hymenoptera of the World: An Identification Guide to Families.* (eds H Goulet JT Huber) pp. 161-278. Agriculture Canada, Ontario: Centre for Land and Biological Resources Research.

Brothers DJ, Lelej AS (2017) Phylogeny and higher classification of Mutillidae (Hymenoptera) based on morphological reanalyses. *Journal of Hymenoptera Research*. 60:1–97.

Brothers DJ, Tschuch G, Burger F (2000) Associations of mutillid wasps (Hymenoptera, Mutillidae) with eusocial insects. *Insectes Sociaux*. 47:201–211.

Harris RA (1979) A glossary of surface sculpturing. *Occasional Papers in Entomology*. 28:1–32.

Houston TF (1975) A revision of the Australian hylaeine bees (Hymenoptera: Colletidae). I. *Australian Journal of Zoology Supplementary Series*. 23:1–135.

Hymenoptera Anatomy Ontology, Hymenoptera Anatomy Consortium. Available from: http://glossary.hymao.org. (accessed 25 August 2018).

Lelej AS (1983) On the velvet ants (Hymenoptera, Mutillidae) of Australia. *Entomologicheskoe Obozrenie*. 62:612–619. [in Russian, English translation: 1984 *Entomological Review*. 62:152–160].

Luz D, Waldren GC, Melo GA (2016) Bees as hosts of mutillid wasps in the Neotropical region (Hymenoptera, Apidae, Mutillidae). *Revista Brasileira de Entomologia*. 60:302–307.

Rayment T (1953) *Bees of the Portland district*. p. 39. Portland Field Naturalists' Club, Victoria, Australia.

Smith F (1879) *Descriptions of New Species of Hymenoptera in the Collection of the British Museum*. pp. i-xxi, 1-240. British Museum, London.

Spessa A, Schwarz MP, Adams M (2000) Sociality in *Amphylaeus morosus* (Hymenoptera: Colletidae: Hylaeinae). *Annals of the Entomological Society of America*. 93:684–692.

Taylor CK, Murphy MV, Hitchen Y, Brothers DJ (2019) Four new species of Australian velvet ants (Hymenoptera: Mutillidae, *Aglaotilla*) reared from bee and wasp nests, with a review of Australian mutillid host records. *Zootaxa*. 4609:201–224.

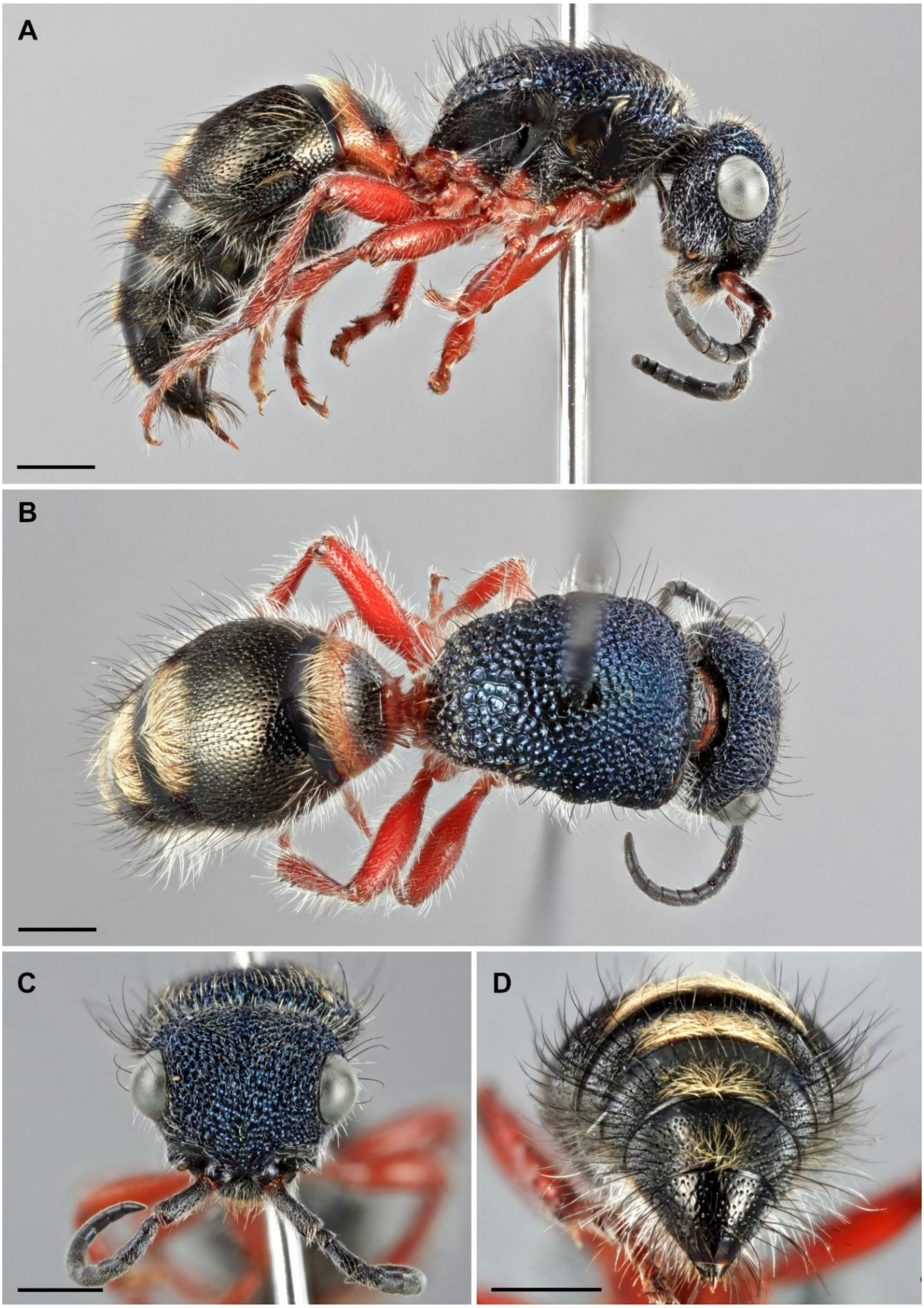

Figure 1. *Ephutomorpha tyla* Hearn, Williams & Parslow sp. nov. holotype female. A, Lateral habitus. B, Dorsal habitus. C, Frontal face. D, paratype female metasoma apex. Scale bar = 1.0 mm.

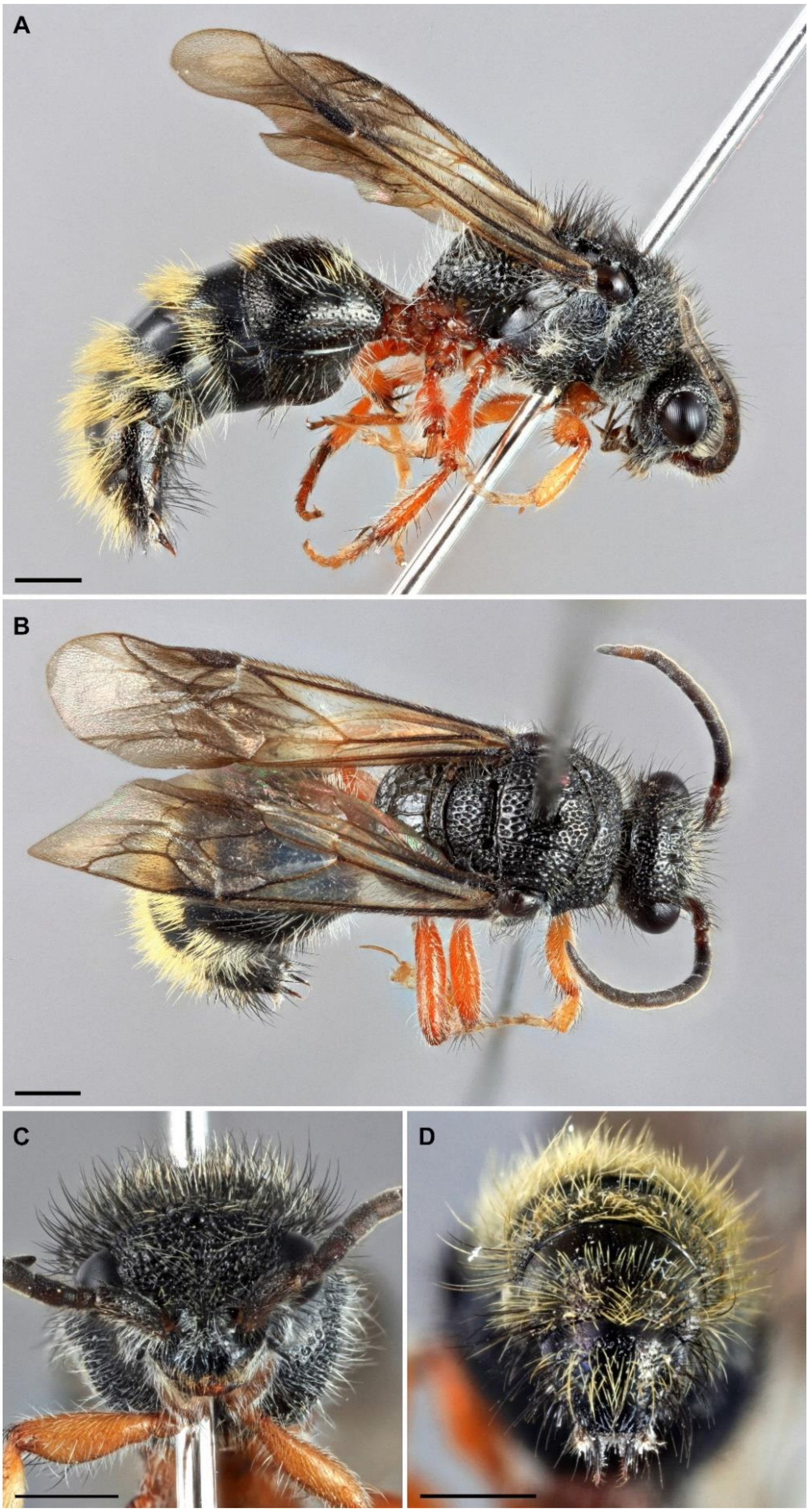

Figure 2. *Ephutomorpha tyla* Hearn, Williams & Parslow sp. nov. paratype male. A, Lateral habitus. B, Dorsal habitus. C, Frontal face. D, Metasoma apex. Scale bar = 1.0 mm.

Figure 3. *Ephutomorpha tyla* Hearn, Williams & Parslow sp. nov. paratype male. A, Ventral genitalia. B, Dorsal genitalia. C, sagittal view with penis valve removed. D, lateral view of penis valve. Scale bar = 0.5 mm.

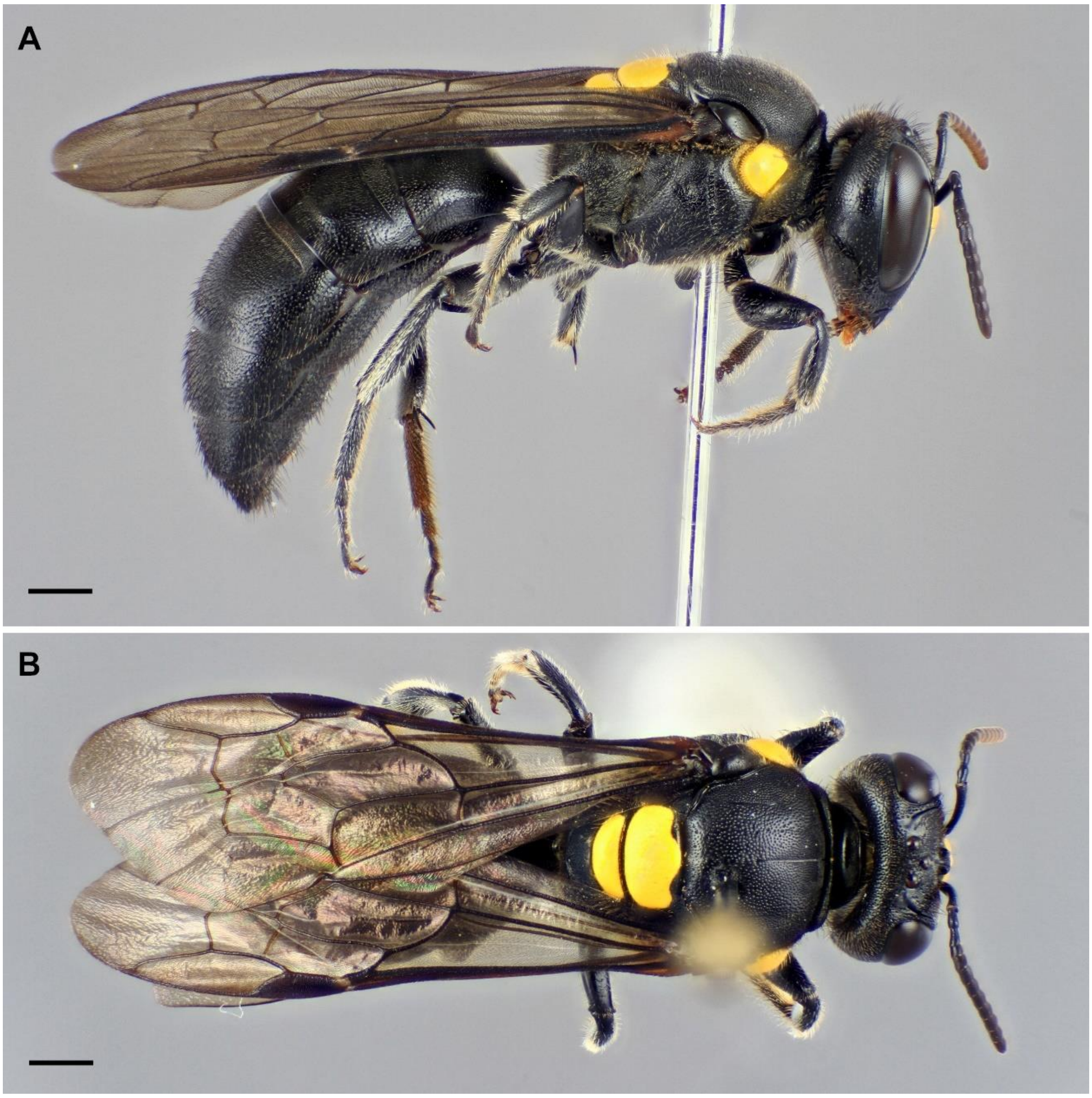

Figure 4. *Amphylaeus morosus***.** ♀. A, Lateral habitus. B, Dorsal habitus. Scale bar = 1.0 mm.

Chapter 3 - Parasitoids of the uniquely social colletid bee *Amphylaeus morosus* (Hymenoptera: Colletidae) in Victoria

This chapter has been published in Memoirs of Museums Victoria

https://doi.org/10.24199/j.mmv.2021.80.10

Lucas R. Hearn[1*], Mark I. Stevens[2, 3], Michael P. Schwarz[1] and Ben A. Parslow[2]

[1]*College of Science and Engineering, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia.*

[2]*Earth and Biological Sciences, South Australian Museum, GPO Box 234, Adelaide, SA 5001, Australia.*

[3]*School of Biological Sciences, University of Adelaide, SA 5005, Australia.*

Corresponding author: Lucashearn7@gmail.com*

3.1 Abstract

Understanding how nest parasites contribute to brood mortality rates in host species is an important step towards uncovering the potential implications for host behaviour. This can be especially important for understanding the evolution of social living, where defence against parasites is often posited as a major benefit of cooperative nesting. Only two parasitoid species have previously been reported for the only known social colletid bee, *Amphylaeus morosus*; namely, the gasteruptiid wasp, *Gasteruption primotarsale,* and the mutillid, *Ephutomorpha tyla*. Here we report six additional parasitoid species of *A. morosus*; the gasteruptiid wasps *G. atrinerve, G. globiceps, G. melanopoda* and *G. cinerescens*, the bombyliid fly *Anthrax maculatus*, and the mutillid wasp *Ephutomorpha* aff. *varipes*. The mechanisms of parasitism for these eight parasitoid species are described in combination with how they operate throughout host brood rearing period and if this affects whether benefits of social nesting vary across the season.

Key words: Bombyliidae, Gasteruptiidae, host-association, Hylaeinae, Mutillidae, Parasitism

3.2 Introduction

Detailed host and parasite association data are important yet rarely explored for primitively social hosts, where defence against parasites is posited to be a driver of cooperative nesting. For insect parasitoids, the value of this information is often emphasised but heavily weighted towards key taxon groups such as Ichneumonidae, Braconidae, Chalcididae, Encyrtidae, Tachinidae and Bombyliidae, that are of economic importance (Heraty 2017). Entomophagous insect associations with non-apid bees largely get overlooked but can have substantial consequences for brood mortality, behaviour and demography of host species (Segers et al. 2016; Smith et al. 2017). Additionally, parasite-host interactions in bees are seldom observed over their full life-history, meaning the full series of parasites attacking a single host is often understated. In the small carpenter bees *Ceratina dupla* Say, 1837, and *C. calcarata* Robertson, 1900, Vickruck et al. (2010) reared a total of eight parasite species from brood cells after sampling nests across a six-month period. Wcislo et al. (2004) similarly sampled nests of the halictine bee *Megalopta genalis* (Meade-Waldo, 1916) across an entire year and reported four brood parasites infiltrating nests. However, Smith et al. (2017) noted that the full affect these brood parasites may have on the social nesting of *M. genalis* is hard to fully assess due to insufficient field data. For analogous species the need for descriptive biological information combined with rigorous sampling across the colony life-cycle is often needed to determine the true diversity and effect of brood parasitism.

The Australian hylaeine bee, *Amphylaeus morosus* (Smith, 1879) (Colletidae: Hylaeinae), is the only species in the hyper-diverse bee family Colletidae that is unambiguously known to exhibit social nesting. Social nesting in this species is very simple, with no known reproductive hierarchies or morphological differentiation between nestmates. It has a semelparous and univoltine life-cycle and may form social or solitary nests within the same population (Spessa et al. 2000). In the Dandenong Ranges, *A. morosus* utilises dead, naturally abscissed fronds of the rough fern tree, *Cyathea australis* to construct linear nests out of the pithy interior. Like many hylaeine bees, *A. morosus* mass provision cells with a mixture of pollen and nectar before ovipositing into the cell.

Spessa et al. (2000) first reported a species of *Gasteruption* wasp, later identified as *Gasteruption primotarsale* (Pasteels, 1957) (Gasteruptiidae) (Parslow et al. 2020a), attacking nests of *A. morosus* in the Dandenong Ranges, Victoria, while a species of mutillid wasp, *Ephutomorpha tyla* Hearn, Williams & Parslow, 2019 (Mutillidae) was recently reported entering nests late in the brood rearing season (Hearn et al., 2019). Spessa et al. (2000) found that social nesting in *A. morosus* seemed to provide only minimal benefits in reducing parasitism by *G. primotarsale*. However, that study did not consider possible defence benefits against other parasites, and indeed no other parasites were identified in that study. *Gasteruption primotarsale* parasitises the earliest brood during nesting period and may therefore only explain potential benefits of social nesting during the early stages of brood provisioning. Additionally, observations of *E. tyla* adults in the nest towards the end of brood-rearing (Hearn et al. 2019) suggests that *E. tyla* may exploit the mature larvae of *A. morosus* towards the end of its nesting period. It is important to understand the full suite of parasites that attack *A. morosus*; if these parasites operate at other times in the nesting period that information will further our understanding of why social nesting in *A. morosus* persists until brood maturation. In south-eastern Queensland, Houston (1969) reported a range of parasites and parasitoids attacking *A. morosus* nests, including the wasps *Agamerion pulchra* (Pteromalidae: Cleonyminae), *Coelopencyrtus* spp. (Encyrtidae), *Gasteruption* sp. (Gasteruptiidae) and an acarid mite. However, in the Dandenong Ranges, little is known about the full range of parasites that might be involved.

Here, we identify eight different parasitoid species that attack *A. morosus,* including novel host records for the *Gasteruption* wasps *G. atrinerve* (Kieffer, 1911), *G. globiceps* Pasteels, 1957, *G. melanopoda* Pasteels, 1957, and *G. cinerescens* Schletterer, 1885, the bombyliid fly *Anthrax maculatus* Macquart, 1846 (Diptera: Bombyliidae) and the mutillid wasp *Ephutomorpha* aff. *varipes* (André, 1895). We examine the specific mechanisms of parasitism and eclosion timings of these parasitoids which have been poorly understood. It is important that the respective host associations are well documented to fully recognise how these multiple parasitoid species may influence the nesting behaviour of the only known social bee in the colletid family.

3.3 Materials and Methods

Nest collections

Nests of *Amphylaeus morosus* were collected from naturally abscissed fronds that haphazardly fall around the base of the rough tree fern, *Cyathea australis* R.Br. Domin. These nests were collected in the Gembrook, Cockatoo and Marysville regions of the Dandenong Ranges, Victoria, Australia. Nests were sampled over five separate collection periods: 4–6th December 2017, 6–8th December 2018, 21–22nd November 2019, 11th January 2020 and 27th February 2020. This region primarily consists of cool temperate montane habitat dominated by tall canopies of *Eucalyptus regnans* F. Muell and *E. viminalis* Labill, with a mixture of ferny, shrubby and grassy understories. Nests were collected either early in the morning, late in the afternoon and/or during periods of light rain when bees were inactive to ensure all colony members were present. Nest entrances were sealed upon collection and all nests were stored in cooled insulated boxes and transported to the laboratory where they were stored at 10 C° until processing.

Parasitism data

Nests were dissected lengthwise and the contents, including adult female *A. morosus*, immatures and nest parasitoids were recorded. Where possible, parasitised cells were carefully extracted from the nests and left to develop in Petri dishes at ambient room temperature. Petri dishes were kept moist by occasionally spraying filter paper rectangles inside each dish with water. Once the specimens had reached adulthood, defined as fully mature and mobile, they were placed in 99% ethanol for preservation and later identification. For parasitoids where the pupal stages form cocoons (e.g. Mutillidae) at least one pupal case from each nest was dissected in coordination with the nest being opened to determine the developmental stage of each immature.

Not all the *Gasteruption* specimens were successfully reared to pupae or adulthood because developmental rates for *Gasteruption* species lagged significantly behind host maturation rates; due to this the specific parasitisation rates for each *Gasteruption* species were unable to be determined. Further, the scarcity of larval descriptions based on morphology for the genus *Gasteruption* made it impossible to discriminate species at the larval stage (Bogusch et al. 2018). Therefore, the parasitisation rates for each species are combined and treated at the genus level '*Gasteruption*'.

Specimen identification

Specimens were examined using a Nikon SMZ1000 stereomicroscope at the South Australian Museum, Adelaide, Australia. Images were taken using a Visionary Digital LK imaging system (Dun, Inc.) with Canon EOS 5DsR camera at Flinders University, Adelaide, Australia. Images were produced using Zerene Stacker (Zerene Systems LLC) software and cropped and resized in Photoshop CS5.

Material examined in this study were initially identified using the following keys: *Amphylaeus morosus* specimens were identified using Houston's (1975) key to Australian hylaeine bees; *Gasteruption atrinerve, G. globiceps, G. melanopoda*, *G. primotarsale* and *G. cinerescens* were identified by B.A. Parslow (South Australian Museum) using Pasteel's (1957) key to the Australian *Gasteruption* and comparison with type material; *Ephutomorpha tyla* specimens were identified by L.R. Hearn by comparing specimens with type material held at the South Australian Museum; and *Anthrax maculatus* was identified by X. Li (Florida Museum). Voucher specimens for the examined species have been deposited at Melbourne Museum (Table S1).

DNA sequencing for the eight parasitoids of *A. morosus* was performed by the Canadian Centre for DNA Barcoding (CCDB) at the Biodiversity Institute of Ontario using standard protocols (Ivanova et al. 2006) (Table S1).

3.4 Results

Nest architecture and composition

In the Dandenong Ranges, females of *A. morosus* construct their nests in dead abscissed fronds of the rough fern tree *Cyathea australis,* by excavating the pithy interiors of the stems to create an unbranched linear tunnel. Nests were on average 212.63 ± 59.39 mm long (mean ± SD; *N* = 156) with nest entrance diameters of 5.65 ± 0.61 mm (*N* = 52). In other regions, nests also occur in the dead flower scapes of *Xanthorrhoea* spp. (L. Hearn pers. obs.). The tunnel is lined with a cellophane-like material, a characteristic common to hylaeine bees, that is thought to act as a waterproofing and antimicrobial agent (Almeida 2008). Provisioning of brood cells generally begins in mid-spring and cells are laid sequentially starting from the distal end of the fern frond towards the proximal end. Brood production can last until mid-summer across the Dandenong Ranges populations, allowing different parasitoid species to attack at different brood development stages (Fig. 1).

Nest architecture in *A. morosus* can be broadly categorised into nests with brood cells interspersed with vestibules and nests with brood cells laid sequentially (Fig. 2). Vestibules are empty spaces containing no provisions or brood and are common in many stem-nesting Hymenoptera (Danks 1971). In *A. morosus,* vestibular cells are frequently found in solitary nests, but nest burrows are fully excavated before the first cell is provisioned. This limitation to nest length once cell provisioning has begun suggests that there may be a trade-off between the number of potential brood cells and constructing vestibular cells to deter nest parasitoids.

Host associations

***Gasteruption* Latreille, 1796 (Fig. 3A).** *Gasteruption* is the most abundant parasitoid of *A. morosus* in terms of nests parasitised, parasitising 16.9% (27/160) nests across all collection periods (Table 1). Of the nests parasitised by *Gasteruption,* only the first three brood cells were found to contain parasitoid immatures. *Gasteruption* parasitised cells were often followed by vestibular cells (11/35 parasitised cells), detritus (9/35) or provisioned cells that failed to develop (4/35).

The larvae of *Gasteruption* are predator-inquilines in the nests of *A. morosus* where they kill the host egg or developing host larva before consuming the entire host provisions (Bogusch et al. 2018;

Parslow et al. 2020a). They then construct using anal secretions, dark semi-cocoons above and below the larvae separating the parasitised cell from the rest of the nest (Eardley and Daly 2007; Malyshev 1968; Westrich 2018). Although some species of *Gasteruption* have been reported to consume the contents of multiple adjacent cells (Donovan 2007; Malyshev 1968; Westrich 2018), *Gasteruption* immatures were only observed to occupy a single host cell in our nests of *A. morosus*.

Adult eclosion of *Gasteruption* occurred far later in the season compared to *A. morosus* (Fig. 4). In the majority of cases, *Gasteruption* brood were still at a late larval stage at the time *A. morosus* cells were reaching adult eclosion. Five species of *Gasteruption* were reared from host nests, *Gasteruption primotarsale* Pasteels, 1957, which has been previously recorded from nests of *A. morosus* (Spessa et al., 2000). The novel host association for *G. atrinerve* (Kieffer, 1911), *G. globiceps* Pasteels, 1957, *G. melanopoda* Pasteels, 1957, and *G. cinerescens* Schletterer, 1885, were determined using adults reared from host nests.

***Anthrax maculatus* Macquart, 1846 (Fig. 3D).** Four nests were found to be parasitised by the bombyliid fly *Anthrax maculatus* across two separate sampling periods. *Anthrax maculatus* was never directly observed ovipositing into or hovering around the nests of *A. morosus*. Yet, developing *A. maculatus* immatures were found occupying both single and consecutive *A. morosus* brood cells, accounting for 24.1% (7/29) and 13.8% (15/109) of the total brood cells across the early summer, December 2018 and mid-summer, January 2020 collection periods, respectively. The pupae of *A. maculatus* were primarily observed occupying cells in the middle of the nests, but in one instance, every cell in the nest contained parasitised immatures. All brood cells parasitised by *A. maculatus* contained late-stage pupae when the nest was opened and, in nests containing both *A. maculatus* immatures and unparasitised host immatures, the host brood cells contained mid to late-stage pupae. The pupae of *A. maculatus* were packed into the host brood cells and eclosed to adults within minutes of being extracted from the brood cell in the nest. The emergence of adult *A. maculatus* therefore appeared to be stimulated by disturbance, but still synchronised with host adult eclosion (Fig. 4).

***Ephutomorpha tyla* Hearn, Williams & Parslow, 2019 (Fig. 3B).** The host association for *Ephutomorpha tyla* has already been described by Hearn et al. (2019). Here we present additional details based on new data. In two nests a single adult female *E. tyla* was observed trying to break through the most proximal cell in the nest, while in a further 21 instances were observed waiting in the nest entrance. *Ephutomorpha tyla* does not appear to specifically target certain nest types, attacking nests with up to 14 brood cells, or nests with only four brood cells interspersed with vestibules. A total of 29 nests contained either parasitised immatures, an adult female *E. tyla* in the entrance, or both. No adult female *A. morosus* were present in 69% (20/29) of these nests.

Parasitised brood cells consisted of papery, brown cocoons which occupied the entirety of the brood cell. As the *E. tyla* immatures developed, the brood cells became darker in colour and increasingly difficult to open. In nine affected nests, all brood cells were parasitised by *E. tyla*, with damaged partitions between cells. The body sizes of male and female *E. tyla* were generally consistent. However, in some cases there were marked differences in body size within each sex.

***Ephutomorpha* aff. *varipes* (André, 1895) (Fig. 3C).** Larvae of *Ephutomorpha* aff. *varipes* were observed as external parasitoids of the mature larvae, prepupae and pupae of *A. morosus* and were observed feeding on ayoung pupae (Fig. 3E). Larvae of *E.* aff. *varipes* remained on their host until mature before producing a light-brown coloured silk-like cocoon and eclosing as an adult. The ectoparasitoid larva of *E.* aff. *varipes* were observed on multiple consecutive *A. morosus* immatures in one nest. The first three brood cells of the parasitised nest contained fully healthy *A. morosus* immatures, whereas the next eight brood cells were occupied by parasitised larvae, all of which eclosed as males. *Ephutomorpha* aff. *varipes* was observed parasitising several species that occupy excised *Cyathea australis* fronds, including another mass provisioning bee, *Hylaeus* sp., and a crabronid wasp, *Pison* sp. While, on numerous occasions, females were observed in the nest entrance of the allodapine bee, *Exoneura robusta* Cockrell, 1922, no parasitic association has been confirmed.

Other enemies

Of the 243 potentially viable *A. morosus* nests collected, 34.2% (83/243) contained no *A. morosus* adults or brood cells and had been superseded or temporarily occupied by various other insect species. Twenty-four of these nests were occupied by *Pison* sp. (Hymenoptera: Crabronidae), two nests were occupied by the pteromalid wasp of the genus *Eupelmophotismus* (Hymenoptera: Pteromalidae), while one nest contained recently eclosed encyrtid wasps from the genus *Coelopencyrtus* (Hymenoptera: Encyrtidae). Five nests contained clerid beetle larvae (Coleoptera: Cleridae), including two instances of nests with *A. morosus* immatures and adult that contained beetle larvae in the nest entrance.

3.5 Discussion

Nest parasitoids are known to have detrimental effects on the survival and productivity of bees (Vickruck et al. 2010; Wcislo et al. 1994). Of the parasitoid species that attack *A. morosus*, the predator-inquilines of the genus *Gasteruption* were the most prevalent, but least detrimental, only parasitising a mean of 1.29 cells per infiltrated nest, with 16.9% of nests being infiltrated. We never observed *Gasteruption* entering nests of *A. morosus*, possibly because our nest collections were restricted to cool weather conditions when insect activity was low, but it is likely that they wait for the adult bee to leave before infiltrating the nest and ovipositing in an open brood cell (Macedo et al. 2012; Parslow et al. 2020a). Parasitisation by *Gasteruption* was only observed in single female nests, which suggests multifemale nests are guarded by one female while other individuals collect provisions, restricting the opportunity for the wasps to oviposit (Parslow et al. 2020a). The extended development time of *Gasteruption* in *A. morosus* nests has been similarly reported in *Gasteruption brachychaetum* Schrottky, 1906a, larvae in nests of *Hylaeus* aff. *guaraniticus* (Schrottky, 1906b) (Macedo et al. 2012). The resulting asynchrony in adult emergence between *Gasteruption* immatures and host could be attributed to delaying their eclosion until hosts have started establishing new nests, which may explain observations of *Gasteruption* pupae in overwintering *A. morosus* nests (L. Hearn pers. obs.). For *Gasteruption*, parasitising the distal nest cells could also be a factor of this extended development period and avoiding being disturbed by emerging bees and other emerging parasitoids. There is limited information on *Gasteruption* host specialisation with a large proportion of records based on single observations (Parslow et. al. 2020a). It is unlikely that *A. morosus* is the only host for *G. atrinerve*, *G. globiceps*, *G. melanopoda*, and *G. cinerescens* as their distributions extend beyond the distribution of *A. morosus* (Atlas of Living Australia 2021; Parslow 2020b; Pasteels 1957).

In contrast, parasitisation by the dipteran *Anthrax maculatus* had a greater effect when invading *A. morosus* nests, parasitising 5.5 brood cells on average across *A. maculatus* infiltrated nests. Previous studies have suggested that adult bombyliid females identify a host nest and oviposit directly into the nest entrance (Bohart et al. 1960). However, there are also reports of adults ovipositing haphazardly onto the ground, leaving the planidium to search for a host nest (Yeates and Greathead 2008). This latter scenario could be likely in the Dandenong Ranges given the high density of *A. morosus* nests that can occur. It was unknown if the larvae of *A. maculatus* feed on both the immatures and provisions in a given cell. Gerling and Hermann (1976) reported early instar larvae of the bombyliid fly, *Xenox tigrinus* (Evenhuis, 1984) (previously *Anthrax tigrinus*), feeding on the pollen bread in *Xylocopa virginica* (Linnaeus, 1771) nests. However, bombyliid fly immatures are also known to feed on the mature larvae of their hosts (Felicioli et al. 2017; Minckley 1989). The delay in adult eclosion by *A. maculatus* compared to its host is consistent with observations by Minckley (1989) of *Anthrax xylocopae* (Marston, 1970) delaying emergence to synchronise with its host. This is because adults are not able to break through the cell partitions and therefore must wait for hosts to emerge

(Gerling and Hermann 1976). This could explain why *A. maculatus* puparium in our current study appeared to be stimulated by disturbance when removed from their cells.

In our study, *Ephutomorpha tyla* was the most abundant and destructive nest parasitoid in terms of both the number of brood cells parasitised and (potentially) adult hosts killed. *Ephutomorpha tyla* was rarely observed in nests with an adult host present, supporting similar reports of mutillids forcefully removing or killing host guards before ovipositing (Brothers et al. 2000). Adult *E. tyla* were generally observed in the nest entrances and on some occasions attempting to break through the cell closest to the entrance, suggesting that *E. tyla* may open the brood cell closest to the entrance to check for host brood before ovipositing. The size differences between reared adults of *E. tyla* are likely due to differences in the consumed host immatures. For instance, in *A. morosus* the males are generally smaller than the females (Spessa 1999). Additionally, the amount of provisions in each brood cell can vary dramatically, which can influence the size of the host immatures and subsequently the ectoparasitoid feeding on it (Brothers 1989). Comparatively, the size differences observed in adults of *E.* aff. *varipes* support the notion that it is likely a parasitoid of multiple insect hosts and this accords with reports by Mickel (1928) that variation in body sizes within mutillid species is linked to them exploiting a range of hosts of varying size. *Ephutomorpha.* aff. *varipes* was observed in nests of numerous different host species including the allodapine bee, *Exoneura robusta* Cockerell, 1922. However, given ‘Brothers’ Rule’ (Brothers et al. 2000) that states mutillids will only attack fully enclosed immatures, it is unlikely that such a condition is universal given that *E. robusta* progressively rears its brood in open linear nests. These observations suggest that *E.* aff. *varipes* is a generalist parasitoid of hosts using *Cyathea australis* as a nesting substrate, rather than any specific hymenopteran host, which accords with the notion that some mutillid species are adapted to specific situations rather than hosts (Brothers 1989).

Attack by wingless parasitoids such as mutillid wasps is uncommon in stem-nesting bees (Ronchetti and Polidori 2020). Michener (1985) suggested that this may be because predators need to search in a three-dimensional space looking for sparsely distributed nesting sites, whereas ground-nesting hymenopteran entrances can be found using a two-dimensional search pattern. In *A. morosus*, the high rates of parasitism might be attributable to the high density of their nesting substrate around mature tree ferns (Groulx and Forrest 2018). This high density of nesting sites may act in a similar system to the ground nesting aggregations of halictid bees, where nesting sites are abundant and parasitism can be prevalent (Wcislo 1996).

The mechanisms of parasitism and bionomy of parasitoids has generally been poorly understood. Our study reveals that parasitoids of *A. morosus* have adapted to host nesting behaviour and also suggests variation in the oviposition chronology of each parasitoid species, with *Gasteruption* wasps ovipositing at the beginning of brood provisioning, *A. maculatus* ovipositing across the middle stages of brood development and the two mutillid species ovipositing in nests at the end of the season. This variation in parasitoid pressure could have implications for understanding the factors driving social nesting in the only known social bee in the family Colletidae.

3.6 Acknowledgements

We thank Terry Houston for his insightful and thorough comments on this manuscript. This project was supported by The Holsworth Wildlife Research Endowment & The Ecological Society of Australia awarded to L.R.H. and in part funded by an Australian Biological Resources Study National Taxonomy Research Grant Programme grant RF217-14 to B.A.P. Nests were obtained under the scientific collection permit 10008943 for Victoria. Thank you to James Dorey Photography for imaging of the parasitised bee pupa.

3.7 References

Almeida EAB (2008) Colletidae nesting biology (Hymenoptera: Apoidea). *Apidologie*. 39:16–29.

André E (1895) Mutillides d'Australie nouvelles ou imparfaitement connues. *Mémoires de la Société Zoologique de France*. 8:475–517.

Atlas of Living Australia - Spatial Portal. AOO and EOO Tool – *Amphylaeus morosus* (Australia) at https://spatial.ala.org.au/. Accessed 21 April 2021.

Bogusch P, van Achterberg C, Šilhán K, Astapenková A, Heneberg P (2018) Description of mature larvae and ecological notes on *Gasteruption* Latreille (Hymenoptera, Evanioidea, Gasteruptiidae) parasitizing hymenopterans nesting in reed galls. *Journal of Hymenoptera Research*. 65.

Bohart GE, Stephen W, Eppley R (1960) The biology of *Heterostylum robustum* (Diptera: Bombyliidae), a parasite of the alkali bee. *Annals of the Entomological Society of America*. 53:425–435.

Brothers DJ (1989) Alternative life-history styles of mutillid wasps (Insecta, Hymenoptera). In: Bruton, M.N. (eds), *Alternative Life-History Styles of Animals*. pp. 279–291. Springer Netherlands, Dordrecht.

Brothers DJ, Tschuch G, Burger F (2000) Associations of mutillid wasps (Hymenoptera, Mutillidae) with eusocial insects. *Insectes Sociaux*. 47:201–211.

Cockerell TDA (1922) Australian bees in the Queensland Museum. *Memoirs of the Queensland Museum*. 7:257–279.

Danks HV (1971) Biology of some stem-nesting aculeate Hymenoptera. *Transactions of the Royal Entomological Society of London*. 122:323–395.

Donovan BJ (2007) *Apoidea (Insecta: Hymenoptera) Fauna of New Zealand 57*. Landcare Research Ltd., Christchurch, New Zealand.

Eardley C, Daly HV (2007) Bees of the genus *Ceratina* Latreille in southern Africa (Hymenoptera, Apoidea). *Entomofauna*. 13:1–94.

Evenhuis NL (1984) Revision of the *Bombylius comanche* group of the new World (Diptera: Bombyliidae). *International Journal of Entomology*. 26:291–321.

Felicioli A, Ambroselli S, Cilia G, Sagona S (2017) Parasitization of a wild and reared population of the solitary bee *Osmia cornuta* Latr. by the parasitoid *Anthrax anthrax* Schrank (Diptera, Bombyliidae): comparison between two types of artificial nest. *Journal of Apicultural Research*. 56:598–605.

Gerling D, Hermann H (1976) The oviposition and life cycle of *Anthrax tigrinus* (Diptera: Bombyliidae), a parasite of carpenter bees (Hymenoptera: Xylocopidae). *Entomophaga*. 21:227–233.

Groulx AF, Forrest JRK (2018) Nesting aggregation as a predictor of brood parasitism in mason bees (*Osmia* spp.). *Ecological Entomology*. 43:182–191.

Hearn LR, Williams KA, Stevens MI, Schwarz MP, Davies OK, Parslow BA (2019) Description and novel host records for a new species of Australian mutillid wasp (Hymenoptera: Mutillidae) from hylaeine bee nests (Hymenoptera: Colletidae). *Austral Entomology*. 58:524–532.

Heraty J (2017) Parasitoid Biodiversity and Insect Pest Management. *Insect Biodiversity*. 603–625.

Houston T (1969) The systematics and biology of Australian Hylaeine bees (Hymenoptera: Colletidae). Ph.D. thesis, University of Queensland, Hertfordshire, St Lucia.

Houston TF (1975) A revision of the Australian hylaeine bees (Hymenoptera: Colletidae). I. Introductory material and the genera *Heterapoides* Sandhouse, *Gephyrohylaeus* Michener, *Hyleoides* Smith, *Pharohylaeus* Michener, *Hemirhiza* Michener, *Amphylaeus* Michener and *Meroglossa* Smith. *Australian Journal of Zoology Supplementary Series*. 23:1–135.

Ivanova NV, Dewaard JR, Hebert PDN (2006) An inexpensive, automation friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes*. 6:998–1002.

Kieffer JJ (1911) Étude sur les Évaniides Exotiques [Hym.] du British Muséum de Londres, *Annales de la Société Entomologique de France*. 80:151–231.

Latreille PA (1796) *Précis des Caractères Génériques des Insectes, disposés dans un order naturel par citoyen Latreille*. Brive: F. Bourdeaux xiv 201:5.

Linnaeus C (1771) *Mantissa plantarum, altera. Generum editionis VI & Specierum editionis II*. Impensis Direct Laurentii Salvii, Holmiae.

Macedo ACC, Cordeiro GD, Alves-dos-santos I (2012) Entering behavior of *Gasteruption brachychaetum* Schrottky (Hymenoptera: Gasteruptiidae) into a nest of *Hylaeus Fabricius* (Hymenoptera, Colletidae). *Revista Brasileira de Entomologia*. 56:325–328.

Marston N (1970) Revision of new world species of *Anthrax* (Diptera: Bombyliidae), other than the *Anthrax albofasciatus* group. *Smithsonian Contributions to Zoology*. 43:5–18.

Macquart M (1846) Diptères exotiques nouveaux ou peu connus. Supplément. *Extrait des Mémoires de la Société royale des Sciences, de l'Agriculture et des Arts de Lille*. 1844:133–364.

Malyshev SI (1968) Genesis of the Hymenoptera. In: *Genesis of the Hymenoptera and the phases of their evolution* pp 3–9. Springer.

Meade-Waldo G (1916) LIII.—Notes on the Apidæ (Hymenoptera) in the collection of the British Museum, with descriptions of new species. *Annals and Magazine of Natural History*. 17:448-470.

Michener CD (1985) From solitary to eusocial: need there be a series of intervening species? *Fortschritte der Zoologie (Stuttgart)*. 31:293–305.

Mickel CE (1928) Biological and taxonomic investigations on the mutillid wasps. *Bulletin of the United States National Museum*.

Minckley RL (1989) Host records and biological notes for two *Anthrax* species in Arizona (Diptera: Bombyliidae). *Journal of the Kansas Entomological Society*. 62:274–278.

Parslow BA (2020b) Systematics and Host Associations of the Australian Gasteruption (Gasteruptiidae: Evanioidea), Ph.D. thesis, Flinders University, Adelaide.

Parslow BA, Schwarz MP, Stevens MI (2020a) Review of the biology and host associations of the wasp genus *Gasteruption* (Evanioidea: Gasteruptiidae). *Zoological Journal of the Linnean Society*. 189:1105–1122.

Pasteels J (1957) Revision du genre *Gasteruption* (Hymenoptera: Evanoidea: Gasteruptionidae). Espèces Australiennes. *Mémoires de l'Institut Royal des Sciences Naturelles de Belgique*. 56:1–125.

Robertson C (1900) Some Illinois Bees. *Transactions of the Academy of Science of St. Louis*. 10:47–55.

Ronchetti F, Polidori C (2020) A sting affair: A global quantitative exploration of bee, wasp and ant hosts of velvet ants. *PLOS ONE*. 15:e0238888.

Say T (1837) Descriptions of new species of North American Hymenoptera, and observations on some already described. *Boston Journal of Natural History*. 1:361–416.

Schletterer A (1885) Die Hymenopteren-gattung *Gasteruption* Latr. (Foenus, aut.). *Verhandlungen der Kaiserlich-Königlichen Zoologisch-Botanischen Gesellschaft in Wien*. 35:267–326.

Schrottky C (1906a) Neue Evaniiden aus Paraguay (Hym.). *Zeitschrift für systematische Hymenopterologie und Dipterologie*. 6:56–62.

Schrottky C (1906b) Contribución al conocimiento de los himenópteros del Paraguay. II. *Anales Científicos Paraguayos*. 6:1–32

Segers FHID, von Zuben L, Grüter C (2016) Local differences in parasitism and competition shape defensive investment in a polymorphic eusocial bee. *Ecology*. 97:417–426.

Smith F (1879) *Descriptions of New Species of Hymenoptera in the Collection of the British Museum*. London: British Museum xxi 240 pp.

Smith A, Harper C, Kapheim K, Simons M, Kingwell C, Wcislo W (2017) Effects of social organization and resource availability on brood parasitism in the facultatively social nocturnal bee *Megalopta genalis*. *Insectes Sociaux*. 65:85–93.

Spessa A (1999) The behavioural and population ecology of an Australian native bee, *Amphylaeus morosus* Smith (Colletidae: Hylaeinae). Ph.D. thesis, Australian National University, Canberra.

Spessa A, Schwarz MP, Adams M. (2000) Sociality in *Amphylaeus morosus*. *Entomological Society of America*. 93:684–692.

Vickruck J, Huber J, Richards M (2010) Natural enemies of the bee genus *Ceratina* (Hymenoptera: Apidae) in the Niagara region, Ontario, Canada. *Journal of the Entomological Society of Ontario*. 141:11–26.

Wcislo WT (1996) Parasitism rates in relation to nest site in bees and wasps (Hymenoptera: Apoidea). *Journal of Insect Behavior*. 9:643–656.

Wcislo WT, Minckley RL, Leschen RA, Reyes S (1994) Rates of parasitism by natural enemies of a solitary bee, *Dieunomia triangulifera* (Hymenoptera, Coleoptera and Diptera) in relation to phenologies. *Sociobiology*. 23:265–265.

Wcislo WT, Arneson L, Roesch K, Gonzalez V, Smith A, Fernández H (2004) The evolution of nocturnal behaviour in sweat bees, *Megalopta genalis* and *M. ecuadoria* (Hymenoptera: Halictidae): an escape from competitors and enemies? *Biological Journal of the Linnean Society*. 83:377–387.

Westrich P (2018) Die Wildbienen Deutschlands vol 821. Eugen Ulmer Verlag, Stuttgart.

Yeates DK, Greathead D (2008) The evolutionary pattern of host use in the Bombyliidae (Diptera): a diverse family of parasitoid flies. *Biological Journal of the Linnean Society*. 60:149–185.

Table 1. Prevalence of parasitoid species in host nests and brood cells at each collection period. Mean parasitised cells calculated from parasitised nests only*.

Nest collection			**Parasitised nests**				**Parasitised cells (total/mean)***			
Year	Total nests	Total cells	*Gasteruption*	*Anthrax maculatus*	*Ephutomorpha tyla*	*Ephutomorpha* aff. *varipes*	*Gasteruption*	*Anthrax maculatus*	*Ephutomorpha tyla*	*Ephutomorpha* aff. *varipes*
Early summer 2017	85	400	16	0	9	0	22/1.38	0/0	37/4.11	0/0
Early summer 2018	6	29	0	2	0	0	0/0	7/3.5	0/0	0/0
Late spring 2019	27	124	7	0	0	0	9/1.29	0/0	0/0	0/0
Mid-summer 2020	26	109	1	2	2	1	1/1	15/7.5	4/2	8/8
Late summer 2020	16	78	3	0	4	0	3/1	0/0	26/6.5	0/0
Total	**160**	**740**	**27**	**4**	**15**	**1**	**35/1.29**	**22/5.5**	**67/4.47**	**8/8**

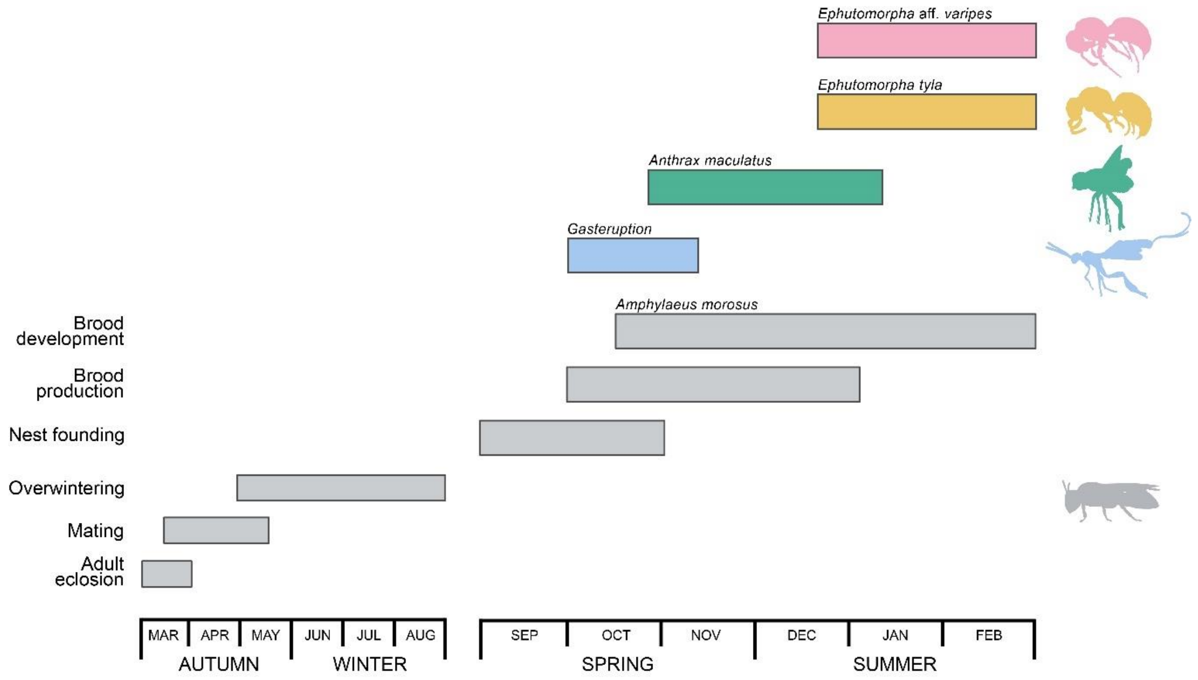

Figure 1. Annual cycle of *Amphylaeus morosus* across the full univoltine, semelparous life-cycle in the Dandenong Ranges, Victoria (grey). The timings of nest parasite attack (coloured) was estimated from rates of occurrence in nests across the brood development period.

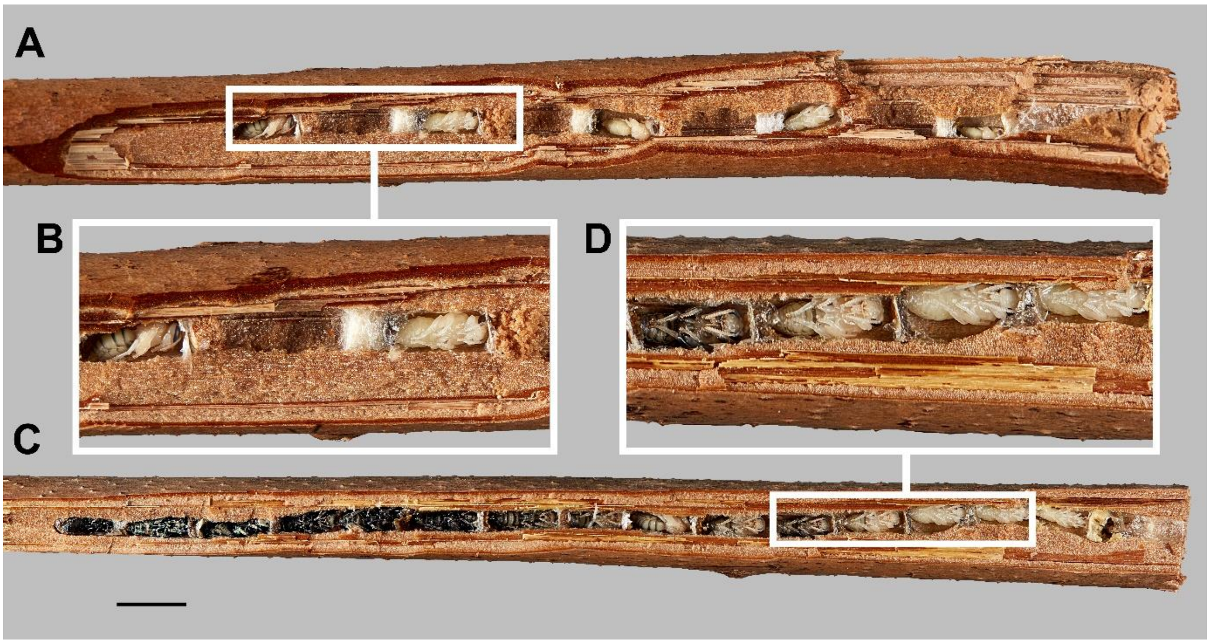

Figure 2. Nest dissections showing the two typical nesting structures for *Amphylaeus morosus* in the Dandenong Ranges. **A** Typical structure of a solitary nest. **B** Close up of brood cells directly followed by a pithy cell plug, interspersed with vestibular cells. **C** Typical structure of a social nest. **D** Close up of brood cells laid sequentially. Scale bar = 10 mm.

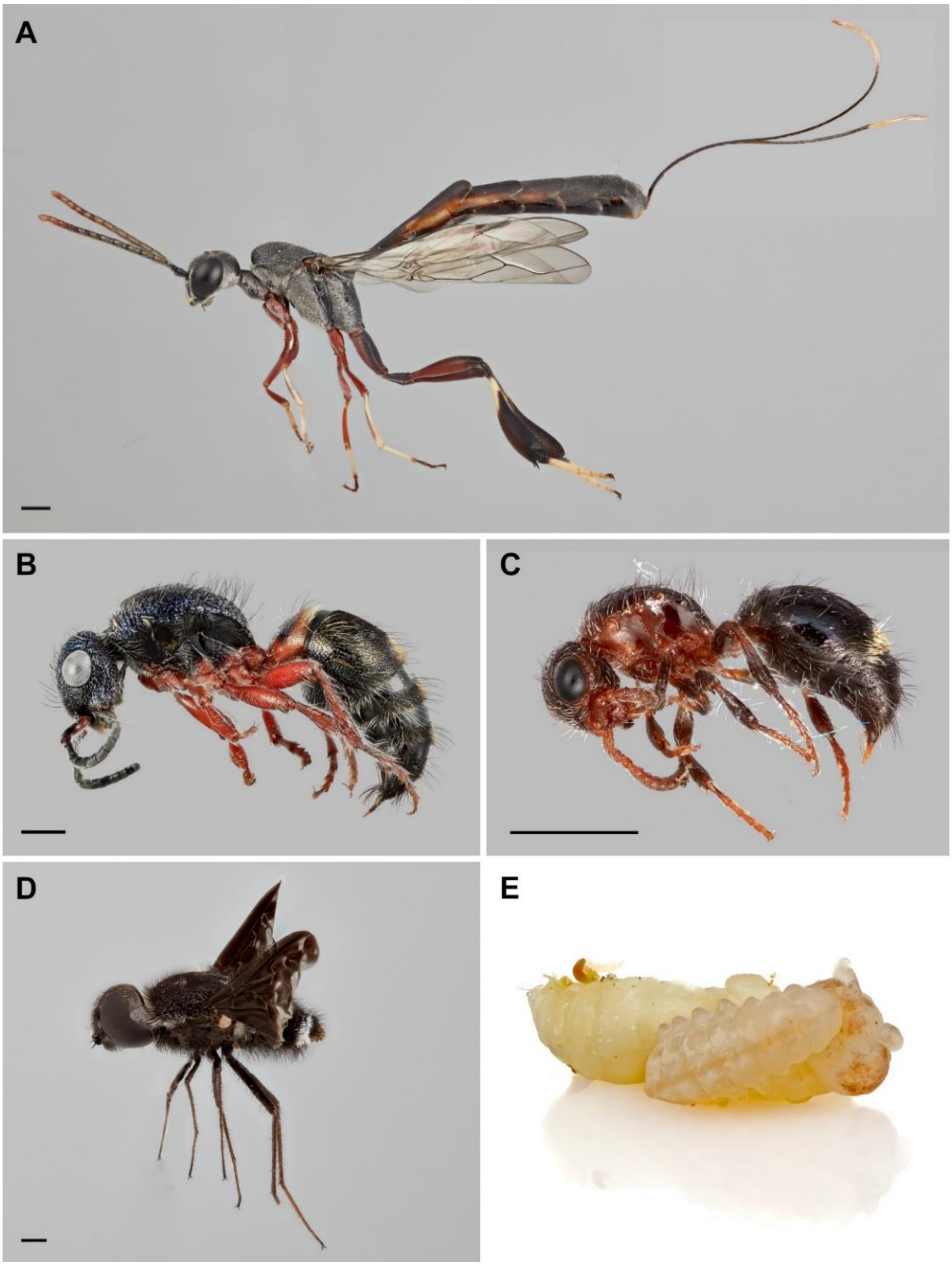

Figure 3. Images of the parasitoids reared from *Amphylaeus morosus* nests. **A** lateral habitus of *Gasteruption globiceps*, **B** lateral habitus of *Ephutomorpha tyla*, **C** lateral habitus of *Ephutomorpha* aff. *varipes*, **D** lateral habitus of *Anthrax maculatus*, **E** *Ephutomorpha* aff. *varipes* larvae feeding on *A. morosus* pupae (photo credit James Dorey, 2020). Scale bar = 1.0 mm.

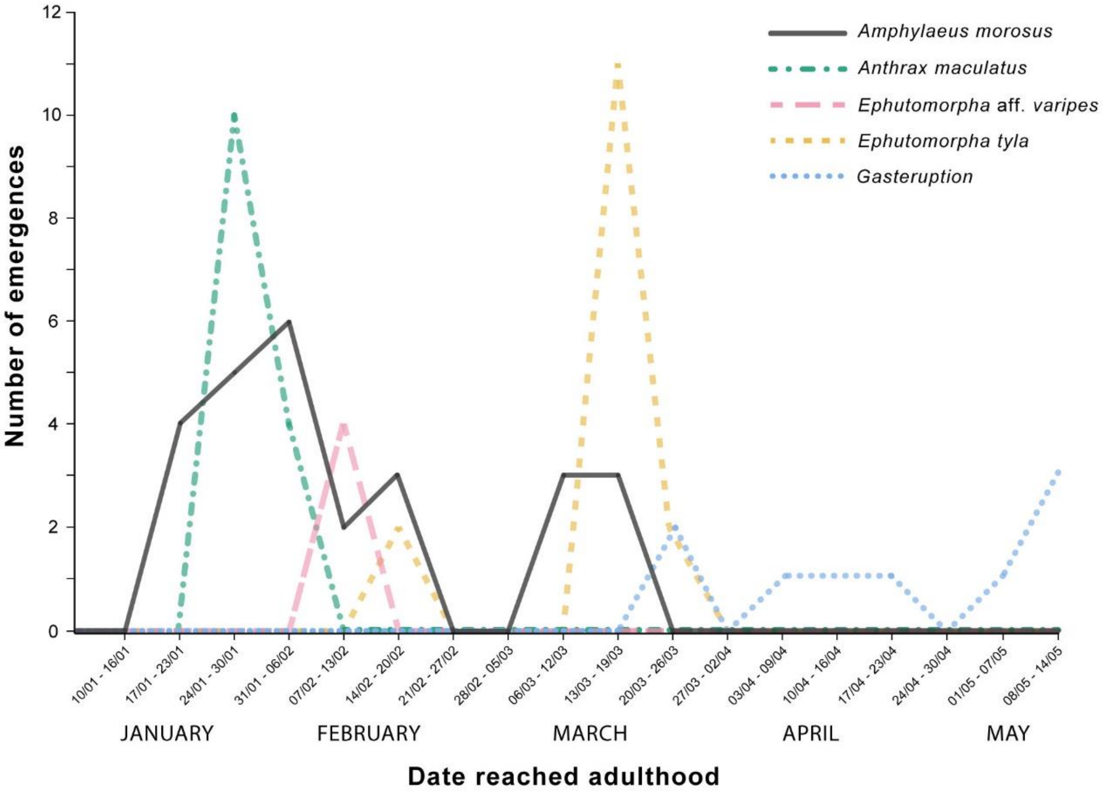

Figure 4. Eclosion phenologies of *Amphylaeus morosus* and its nest parasites. Emergence observations are only reported for the provisioning and rearing phase during 2019. Host emergences are only reported for nests containing parasitised brood.

3.8 Supplementary material

Appendix

Table S1. Table showing BOLD sequencing reference number and voucher number for deposited material. All material deposited at Melbourne Museum.

Family	**Species**	**Melbourne Museum Voucher number**	**BOLD reference number**
Gasteruptiidae	*Gasteruption primotarsale*	HYM-97074	OZBOL2593-21
Gasteruptiidae	*Gasteruption globiceps*	HYM-97075	OZBOL2607-21
Gasteruptiidae	*Gasteruption atrinerve*	HYM-97076	OZBOL2606-21
Gasteruptiidae	*Gasteruption cinerescens*	HYM-97077	OZBOL2605-21
Gasteruptiidae	*Gasteruption melanopoda*	HYM-97078	N/A
Bombyliidae	*Anthrax maculatus*	DIP-2590	OZBOL2594-21
Mutillidae	*Ephutomorpha tyla*	HYM-97079	OZBOL2596-21
Mutillidae	*Ephutomorpha* aff. *varipes*	HYM-97080	OZBOL2599-21

Chapter 4 – Temporal dissonance between group size and its benefits requires whole-of-lifecycle measurements

Lucas R. Hearn[1*], Ben A. Parslow[2], Mark I. Stevens[2, 3] and Michael P. Schwarz[1]

This chapter has been published in Behavioral Ecology

https://doi.org/10.1093/beheco/arac025

[1]*College of Science and Engineering, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia.*

[2]*Earth and Biological Sciences, South Australian Museum, GPO Box 234, Adelaide, SA 5001, Australia.*

[3]*School of Biological Sciences, University of Adelaide, SA 5005, Australia.*

Corresponding author: Lucashearn7@gmail.com*

4.1 Abstract

The benefits of living in groups drive the evolution of sociality, and these benefits could vary across a life-cycle. However, there may be experimental problems in linking group size at one time in a life-cycle to benefits that only become apparent later on when group size has changed, leading to what we call "temporal dissonance". In the only known social colletid bee, *Amphylaeus morosus*, parasite pressures arise at various times throughout the life-cycle from different parasitoid species. *Amphylaeus morosus* is impacted by eight different parasitoid species operating at different host-colony phenology phases, including five species of *Gasteruption* wasps, a bombyliid fly and two mutillid wasp species. We found that, as the reproductive season progressed, the number of host adults in a nest declined, often to zero, but the presence of even one adult host female during late brood-rearing stages appeared to offer substantial brood protection against mutillids. We propose that the apparent benefits of colony size at one point in time may not reflect the benefits that become apparent at a later point in the season, leading to a temporal dissonance between group size and its later fitness benefits. We also show that *A. morosus* is strongly protogynous, with variation in parasitoid pressure across the reproductive phenology distorting operational sex ratios away from initial investment ratios. Combined, our data suggest that seasonal variation in parasitoid pressure may have major consequences for understanding social evolution, but these kinds of consequences are largely unexplored in current studies of insect social evolution.

Key words: Parasitism, Social evolution, Sex ratio, Colletidae, Mutillidae, Gasteruptiidae

4.2 Introduction

Parasitism is a major source of brood mortality and can incur substantial fitness costs. For species that rear offspring in a nest, that nest becomes a concentrated resource for parasites. During this brood rearing period, this concentration of resources suggests that hosts would receive fitness benefits by investing in nest defence, both spatially and temporally: a host nest therefore becomes a rich resource for enemies, but one that can be locally defended. This local defensibility is posited as a key driver in the evolution of social groups if cooperative defence by more than one adult is effective (Lin and Michener 1972; Michener 1974; Andersson 1984; Wcislo 1997). However, exactly how extrinsic factors such as parasitism influence social nesting is still poorly understood (Soucy 2002; Prager 2014; Smith et al. 2017).

Variation in parasitism rates and infiltration mechanisms are strongly influenced by various host traits, including population density, social behaviour and nesting behaviour (Farzan and Yang 2018; Groulx and Forrest 2018). However, one factor that can get overlooked when attempting to interpret parasitism rates is brood rearing phenology of the host. Parasitoids and nest predators generally prefer specific host stages, which can lead to interspecific variation in parasitism along a temporal scale (Hearn et al. 2021; Hood et al. 2021). Temporal variation in parasitism has been reported for the social ground nesting bee, *Lasioglossum malachurum* (Kirby, 1802), where nest enemies exploited different ‘windows of opportunity’ over the nesting cycle based on what host resources were present (Polidori et al. 2009). In a very different example, socially parasitic bees in the genus *Inquilina* Michener, 1961 delay oviposition in their host colonies until after hosts have laid a substantial number of eggs, presumably a strategy to mask parasite eggs with olfactory cues from the accumulated host brood (Smith and Schwarz 2006).

Variation in parasitoid strategies can alter the risk of parasitism across different stages of the host life-cycle. For mass provisioning bees, this can lead to a significant shift in behavioural strategies, which may include bet-hedging and more commonly, social nesting (Lin 1964; Smith et al. 2003; Mikát et al. 2016). While social nests containing two or more individuals may be better equipped to defend against nest parasites compared to solitary nests, they can incur high costs through sacrificing reproductive opportunities (Hogendoorn and Velthuis 1999; Rehan et al. 2011; Shell et al. 2018). Furthermore, if parasitoid pressure varies across the host phenology then it could lead to temporally varying pressures on host defences. To minimize these associated costs, colonies could potentially direct anti-parasitoid strategies to seasonal or host-phenology periods when the risk of parasitism is highest. For many bee and wasp species the greatest risk of parasitism has been reported to be at the end of brood development, when the parasitism rate of highly destructive invaders such as mutillids is greatest (Brothers et al. 2000; Polidori et al. 2010; Rocha-Filho and Melo 2017), which has also been reported for mutillids parasitising *Amphylaeus morosus* (Smith, 1879) (see Hearn et al. 2019).

One largely neglected issue in studies of parasitism on social insects is whether phenological variation in parasitism rates will distort host sex ratios. In most situations species are expected to invest equally in sons and daughters, at a population level, even if unequal survival of male and female offspring leads to operational sex ratios that are biased (Charnov 1982; Hardy 2002; West 2009). However, if parasitoid pressure varies across host brood-rearing phenology, we might expect host species to react by adjusting the order in which they produce sons and daughters in a way that minimizes the risk to their most costly investments (West et al. 2005; Seidelmann 2006). Most non-eusocial bees provide larger provisions to daughters compared to their sons (Knerer and Schwarz 1976; Frank 1995; Bull and Schwarz 2001), so in sequentially provisioning species we might expect daughters to be produced in cell positions that have lower risks of parasitism. Whilst this may not affect sex allocation *per se*, it may impact operational sex ratios once brood reach maturity.

Amphylaeus morosus (Colletidae: Hylaeinae) is the only unambiguously known social bee in the diverse bee family Colletidae and exhibits a very simple form of social behaviour (Spessa et al. 2000). *Amphylaeus morosus* is univoltine and the females mate prior to overwintering in nests in abscised tree fern fronds (*Cyathea australis* (R.Br.) Domin). In early-spring females undertake further nest construction/maintenance and start provisioning brood cells in a linear sequence by mid-spring (Fig. 1A). The nests are lined with a cellophane-like secretion which is used to create brood cells containing liquid provisions (regurgitated nectar and pollen mixture), and these are often separated by vestibules (empty cells) in solitary nests (Hearn et al. 2021). Eight nest parasitoids are known for *A. morosus* in the Dandenong Ranges, Victoria, Australia; wasps of the genus *Gasteruption* Latreille, 1796 (Gasteruptiidae), *G. atrinerve* (Kieffer, 1911), *G. globiceps* Pasteels, 1957, *G. melanopoda* Pasteels, 1957, *G. primotarsale* Pasteels, 1957, and *G. cinerescens* Schletterer, 1885, the bombyliid fly *Anthrax maculatus* Macquart, 1846 (Diptera: Bombyliidae) and two mutillid (Mutillidae) wasp species *Ephutomorpha tyla* Hearn, Williams & Parslow, 2019 and *Ephutomorpha* aff. *varipes* (André, 1895). *Gasteruption* wasps will wait until the host has left before ovipositing into open cells, where their larvae consume the host eggs and provisions (Spessa et al. 2000; Macedo et al. 2012; Parslow et al. 2020). No direct observations are available for adult *A. maculatus* activity in or around nests of *A. morosus*, despite observations of parasitised brood, however, reports for other bombyliid species have observed larva feeding on host provisions, egg or larva suggesting a potentially wide scope of attack (Gerling and Hermann 1976; Minckley 1989). The mutillid wasps *E.*

tyla and *E*. aff. *varipes* will attack host offspring after larval feeding has finished, which is generally later in the season in mid-summer (Hearn et al. 2021).

We use these species involving the only demonstrably social bee species in the family Colletidae, *A. morosus*, and its parasitoids, using nests collected across four years in the same study region. We investigate if the risk of parasitism changes throughout the reproductive season and predict that such parasitic pressures will influence brood production patterns during periods of heightened parasitism. Our study is a rare documentation of a social species' phenology across the full life-cycle and has important implications for the benefits of social nesting across a semelparous and univoltine life-cycle.

4.3 Materials and Methods

Nest collections

In total, 160 *A. morosus* nests containing 740 brood cells were collected from abscised fronds of the rough fern tree, *C. australis* in the Gembrook (37.9500° S, 145.5410° E), Cockatoo (37.9350° S, 145.4920° E) and Marysville (37. 5151° S, 145.7631° E) regions of the Dandenong Ranges, Victoria, Australia. Nests were collected across the full reproductive season (November to February) over five separate collection periods during early austral summer 2017 (December 2017, number of nests N = 85) and again in 2018 (December 2018, N = 6), late spring 2019 (November 2019, N = 27), mid-summer 2020 (January 2020, N = 26) and late summer 2020 (February 2020, N = 16; Table S1). Nests were collected early in the morning, late in the afternoon and/or during periods of rain when bees were inactive to ensure all colony members were present. Nests were stored on crushed ice in cooled insulated boxes and transported back to Flinders University where they were stored at 10 C° until opening.

Colony data

Nests were processed within 24 hours after being returned to Flinders University, and opened longitudinally up to the first brood cell, with all adult *A. morosus* and additional contents within the nest entrance removed and placed in 99% ethanol. The maturity of the brood cell closest to nest entrance was examined to determine if the brood were at a pupal stage where they could be visually sexed. Because *A. morosus* construct brood cells in a linear array, starting from "cell 1" (first brood laid – furthest from the nest entrance) to a maximum of "cell 16" (last brood laid – closest to the nest entrance) in the largest of nests, the cell closest to the nest entrance is expected to be the least mature brood. In this species, brood laid earlier are generally larger and take longer to develop compared to recently laid brood (Spessa 1999). The cell closest to the entrance will therefore give an indication of brood maturity for the nest. Immature brood for a nest were left in their cells in a sealed container and reared to pupation at ambient room temperature (~22 °C).

Parasitization rates

Nests were completely opened once the brood had reached adult or callow stage, as indicated by the brood cell closest to the nest entrance. The position of each parasitised cell within the nest was recorded. Parasite species were identified using photographic and morphological aids described in detail by Hearn et al. (2021). Parasitoid species were identified as follows: *Gasteruption atrinerve*, *G. globiceps*, *G. melanopoda*, *G. primotarsale* and *G. cinerescens* were identified by B.A. Parslow (South Australian Museum (SAM)) using keys to the Australian *Gasteruption* in Pasteel (1957) and Parslow (2020) and comparison with type material; *Ephutomorpha tyla* specimens were identified by L.R. Hearn by comparing specimens with type material; and *Anthrax maculatus* was identified by X. Li (Florida Museum). Voucher specimens of all parasitoid species are deposited at Melbourne Museum (see Hearn et al. 2021).

If parasitoid brood were initially difficult to identify due to their immature status, they were left in the nest to develop to pupal or adult stages. As all observed parasitoid brood only consume the contents of a single cell (Hearn et al. 2021), the removal of parasitised brood from the nest for identification purposes did not influence measures of parasitization rates (Fig. S1).

Statistical analyses

Amphylaeus morosus has a univoltine and semelparous lifecycle with a reproductive phenology that approximately spans a four-month period from November (mid-spring) to February (late summer). To determine how parasitoid pressure changed between the beginning and end of the reproductive season we divided the reproductive season into two periods that roughly corresponded to early or late periods in the reproductive phenology. We classified nests collected in November and December as ‘early’ and all nests collected in January and February as ‘late’. Only nests containing at least one brood cell were used in our analyses.

Statistical analyses were performed using SPSS version 27.0 (IBM, Armonk, NY) and R version 4.0.4 (R Core Team, 2018). Where data did not meet assumptions of homoscedasticity, non-parametric tests were used. We used Mann-Whitney U tests and Kruskal-Wallis tests to determine statistical significance of the proportion of parasitised brood at different cell positions and different periods of the reproductive season and a chi-square goodness-of-fit to determine if the sex ratio significantly differed from a hypothesised 1:1 ratio (Sokal and Rohlf 1969). We used a generalised linear model (GLM) with a binomial distribution and logit link to compare the probability of a cell being parasitised at each cell position within the nest (where 0 = not parasitised, 1 = parasitised). Values are reported as mean ± standard error unless otherwise stated.

4.4 Results

Variation in attack patterns

Across cell positions

We examined brood parasitization across cell position to test the relationship between the order of cells and their rates of parasitization (Fig. 2). The sequential order of parasitised cells for each parasitoid species differed significantly (Kruskal-Wallis: $H = 51.325$, d.f. = 3, $P < 0.001$). Non-parametric Dunn-Bonferroni post-hoc tests revealed that parasitization of the first three cells by *Gasteruption* occurred far more frequently compared to the three other parasitoid species, whereas there was no difference in cell placement between *A. maculatus*, *E. tyla* and *E.* aff. *varipes* (Table S2). Of the 160 nests, 11 were completely failed due to parasitisation (6.88%) (Fig. 2).

Across reproductive season

To determine if there was a higher success rate laying brood earlier or later during the reproductive season, we compared parasitization rates from nests collected in November and December (early reproductive stage) and nests collected in January and February (late reproductive stage). Overall, there was no difference in the number of parasitised nests between each stage of the reproductive season (Early: 29.82% ± 45.9, Late: 30.77% ± 46.8; Mann-Whitney: $U = 2202.0$, $P = 0.912$), however nests collected later in the reproductive season had a significantly higher proportion of parasitised cells (Early: 13.5% ± 34.2, Late: 30.11 ± 45.9; Mann-Whitney: $U = 40858.0$, $P < 0.001$; Table 1). There was a higher proportion of cells parasitised by *Gasteruption* in nests collected early in the reproductive season compared to nests collected late (Early: 5.69% ± 23.2, Late: 1.61% ± 12.6; Mann-Whitney: $U = 47011.5$, $P = 0.023$; Fig. 3). Conversely, in the nests collected late in the reproductive season there was a higher proportion of parasitised cells from *A. maculatus* (Early: 1.33% ± 11.5, Late: 8.06% ± 27.3; Mann-Whitney: $U = 45709.5$, $P < 0.001$), *E. tyla* (Early: 6.45% ± 24.6, Late: 16.13% ± 36.9; Mann-Whitney: $U = 44268.0$, $P < 0.001$) and *E.* aff. *varipes* (Early: 0.0% ± 0.0, Late: 4.30% ± 20.3; Mann-Whitney: $U = 46903.0$, $P < 0.001$) compared to earlier in the reproductive season (Fig. 3).

Adult female presence

Nests with multiple nestmates at the time of collection were infrequent and were only collected in November and December (2.5% of nests). Mean colony size (number of adult females per nest) was highest during December (0.82 ± 0.04) and declined as the reproductive season progressed (February: 0.43 ± 0.14). Conversely, as colony size decreased across the reproductive season, the number of cells parasitised by both *E. tyla* and *E.* aff. *varipes* showed a monotonic increase (Fig. 4). We examined whether the presence or absence of an adult *A. morosus* female was associated with both the number and the proportion of parasitised cells per nest. Nests containing no adult *A. morosus* females had a significantly higher number and proportion of parasitised cells compared to nests with an adult present (number of cells: Mann-Whitney: U = 1997.5, P = 0.002; proportion of cells: Mann-Whitney: U = 1851.5, P = 0.003). Nests later in the season with at least one adult female experienced significantly less parasitism compared to nests with no adult female (Mann-Whitney: U = 114.0, P = 0.017), whereas there was no significant difference early in the reproductive season (Mann-Whitney: U = 1156.0, P = 0.156; Fig. S2).

Sex ratio

Sex ratios for individual nests were significantly correlated with clutch size (Spearman’s rank coefficient: r = 0.562, $P < 0.001$), such that the proportion of male brood increased with clutch size (Fig. S3). Numerical sex ratios showed an increasing sigmoidal trend with cell position (Fig. 5). Female brood were primarily located in the first series of cells that were furthest from the nest entrance (cell positions 1-7; numerical ratio = 0.337, chi-square: χ^2 = 28.015, $P < 0.001$), whereas the numerical ratio for the later cells (8-16) was exclusively male (numerical ratio = 1.0, chi-square: χ^2 = 59.0, $P < 0.001$).

To determine if the sex ratio at a given cell position was associated with parasitization rates, we performed a multiple linear regression using a stepwise approach. Parasitization significantly predicted the sex ratio at a given position within the nest ($F_{(2,13)}$ = 30.248, $P < 0.001$, R^2 = 0.823; Fig. 5).

Risk of parasitism

To assess the total incidences of parasitization across cell positions, we used a binary logistic regression to calculate the probability of a cell being parasitised based on the observed presence or absence of parasitization at each cell position for nests collected at early and late stages of the reproductive period (Fig. 5). The predicted probabilities for early and late reproductive periods generated from the binary logistic regression equation showed opposing trends across cell position. Early in the reproductive season, the potential for parasitization was greatest in the first cell and gradually decreases across cell position. In comparison, late in the reproductive season there was a weakly sigmoidal trend increasing from cell positions 1 to 16.

We used a generalised linear model to test the relationship between the predicted probabilities of the binary logistic regression and each parasitoid species. There was only a significant relationship between the predicted probabilities and parasitization by *Gasteruption* early in the reproductive season (GLM, $\chi^2 = 38.512$, $P < 0.001$), whereas the relationship between the predicted probabilities and observed parasitization was only significant for both mutillid species later in the reproductive season (*E. tyla*: GLM, $\chi^2 = 16.039$, $P < 0.001$; *E. aff. varipes*: GLM, $\chi^2 = 12.135$, $P < 0.001$). There was no significant relationship for *A. maculatus* in either stage of the reproductive season (early: GLM, $\chi^2 = 0.059$, $P = 0.809$; late: GLM, $\chi^2 = 1.392$, $P = 0.238$).

4.5 Discussion

Our study aimed to explore how parasitization rates vary across the reproductive period of the uniquely social colletid bee *Amphylaeus morosus* which is likely to represent an early stage in social nesting behaviour within bees. In particular, we explored whether any such variation may influence the benefits of social nesting, and whether it may also impact operational sex ratios.

Diversity of parasites

Assessments of parasitization rates in bees are most frequently taken at a single point in the life-cycle and generally towards the end of the reproductive phase when brood are reaching maturation and the rate and diversity of parasitism is highest (Seidelmann 1999; Smith et al. 2017; Díaz et al. 2021). This could result in the under estimation of true parasitoid diversity, especially for bees with multivoltine or staggered life-cycles.

For bees, there is a tendency for a greater number of parasitoid species to be associated with bees in large ground nesting aggregations (Polidori et al. 2009; Lienhard et al. 2010) and in an earlier study Michener (1985) suggested that ground nesting bees were likely to suffer greater parasite and predator pressure than stem nesters. The parasitoid communities recorded from stem-nesting bees generally have a lower species diversity compared to their ground nesting counterparts, despite their higher mortality rates (Danks 1971; Michener 1985; Wcislo 1996; Minckley and Danforth 2019). However, our observations have shown the suite of parasites associated with *A. morosus* is extensive and akin to those found in ground nesting bees (e.g. Wcislo et al. 1994; Polidori et al. 2009; Lienhard et al. 2010; Minckley and Danforth 2019). Understanding the bionomic data of social hosts and their nest parasitoids is important when generating assumptions of the behavioural influences they may cause hosts. Our results show that for *A. morosus*, the temporal windows at which specific parasitoids might attack could influence the behavioural strategies used to deter or minimise the impact of parasitization.

Variation in parasitoid pressure

The difference in parasitization rates across the reproductive season in our study was only observed in terms of cells parasitised rather than the number of nests parasitised. The proportion of brood cells parasitised increased significantly as the reproductive season progressed. This discrepancy can be attributed to the main source of parasitism early in the reproductive season, with *Gasteruption* only attacking the first three cells in a nest (Fig. 2). Comparatively, *Anthrax maculatus* and the two mutillid species were able to make their way through sequential cells, with *Ephutomorpha tyla* sometimes extirpating entire nests (Yeates and Greathead 1997). We also found that the higher proportion of parasitised cells later in the reproductive season was generally a function of the parasitoid species that were active during this period rather than an accumulation of parasitoids over time.

The distribution of parasitised cells in the linear nests of *A. morosus* combined with the disproportionate frequency of species at each stage of the reproductive period indicates there is asynchrony in the attack phenologies of each parasitoid species. The pattern of *Gasteruption* wasps attacking early in the reproductive season, *A. maculatus* attacking midway through the season and both mutillid wasp species at the end of the reproductive season, reflects the requirement of different parasitoid species in regard to host stage preference (Gerling and Hermann 1976; Brothers 1989; Parslow et al. 2020). Polidori et al. (2009) found that enemies of the sweat bee *Lasioglossum malachurum* exploited different host resources leading to variation in attack patterns. The cuckoo bee, *Sphecodes monilicornis* (Kirby, 1802) targeted fresh host provisions, while the mutillid wasp *Myrmilla capitata* (Lucas, 1849) attacked more mature brood later in the life-cycle. Interestingly, frequent observations of a *Gasteruption* wasp and bombyliid fly at the nesting sites of *L. malachurum* were recorded, although no confirmed association was reported (Polidori et al. 2009). In our system, *Gasteruption* wasps target cells early in the reproductive season as their larvae feed on host eggs and nest provisons in the form of pollen and nectar (Macedo et al. 2012; Parslow et al. 2020); *A. maculatus* has a wider scope of attack and may infiltrate host cells containing provisions, egg or larval stage brood, as has been reported for related species (e.g. Gerling and Hermann 1976; Minckley 1989) and, the larvae of the two mutillid species are ectoparasitoids on late-stage larvae or pupae so attack nests later in the reproductive season (Brothers 1989; Brothers et al. 2000).

Risk of parasitization

Risk across reproductive season

We found that the greatest risk of brood cell parasitization came at the beginning of the provisioning phase from gasteruptiid wasps infiltrating unattended nests, and then again later in the season when the chance of nest extirpation by mutillid wasps was high. These varying influxes of parasitoid pressure create conflicting challenges for both parasitoid and host throughout the reproductive period (Fig. 1B). Parasitoids attacking the earliest stages in brood provisioning will encounter fewer brood cells that will primarily contain pollen and nectar resources, so need to be able to extract nutrients from plant material (Lundgren 2009). These parasitoids will generally have to contend with a higher number of host defenders (Lienhard et al. 2010). Conversely, parasitoids attacking towards the end of the reproductive season are more likely to find full nests containing mature host brood with senescent host females or no host defenders (Fig. 1B).

Risk associated with adult presence

At early stages of the reproductive season, when colonies may contain multiple females, group size might not provide strong defence against gasteruptiid wasps, as only a few cells containing provisions are attacked. However, as time goes on those larger colonies are more likely to have at least one surviving female when brood are reaching maturity and that single surviving female might be important for defence against mutillids and nest extirpation (Smith et al. 2003).

Adjustments in sex ratio

The variation in parasitoid pressure across the reproductive phenology may prompt host mothers to adjust the placement of the more costly sex. The ability for daughters to re-use their natal nest has been shown to influence the formation of multifemale nests in *A. morosus* and this may impact how mothers skew sex ratio across the reproductive season (Spessa et al. 2000). For instance, in nests of *A. morosus*, females are generally larger in size and are provisioned in the first five cells of a nest, furthest from the entrance (Spessa 1999). While these cells are still highly vulnerable to parasitization by *Gasteruption* wasps, it may be more beneficial for a mother to lay early in the season when foraging trips are likely to be more efficient and the prevalance of more destructive parasitoids is lower (Goodell 2003; Hearn et al. 2021). Conversely, later in the reproductive period, mothers might attempt to maximise fecundity by investing in the cheaper sex when environmental conditions are poorer and the risk of parasitism is high (Torchio and Tepedino 1980; Fox and Czesak 2000). In larger *A. morosus* nests, mothers shift the sex ratio towards males as the reproductive period progresses. This is similar to maternal investment patterns in the Red Mason bee, *Osmia rufa* (L.) that reduce their investment costs over the nesting season (Ivanov 2006; Seidelmann et al. 2010). This strategy allows mothers to minimise fitness costs through adjustments in brood sex placement when environmental conditions are poor.

Consequences for social nesting

A potentially confounding factor of census-based studies such as this one, is the underestimation of the number of females in a nest at a given time throughout the reproductive season due to premature abandonment or mortality. While we are unable to recapture any females that may have been in the nest at the start of the season, previous collection data by Spessa et al. (2000) suggests that social nesting consists of no more than three adult females in a nest at the start of the reproductive season.

It is likely that the changing pressures on host defences over time could lead to cooperative nest defence at key points in the life-cycle. Parasitoid pressure is generally thought to be an important selective pressure for promoting social nesting (e.g. Lin and Michener 1972; Michener 1974; Rehan et al. 2011). For *A. morosus*, the variation in parasitoid pressure across the reproductive season might mean there are greater benefits to social nesting later in the season. In some social halictine species, nest guards have been shown to deter mutillid invaders by either blocking the nest entrance or through extended fights (Brothers et al. 2000; Bergamaschi et al. 2010). Spessa et al. (2000) found that parasitization rates by *Gasteruption* wasps did not differ enough between solitary and social nests of *A. morosus* to suggest that there is a protective benefit to cooperative nesting. However, those collections only detected the threat of a single *Gasteruption* species and so the full extent of parasitism was not realised. The extensive threat of parasitism to *A. morosus* nests across the entire reproductive phenology could select for cooperative nesting during periods of heightened parasitoid pressure. Additionally, the presence of two females early in the reproductive period might act as an insurance policy, increasing the chance that at least one female is still present at the end of the reproductive period, similar to the principles suggested by the assured fitness returns theory (Queller 1989; Gadagkar 1990).

These findings raise important questions relating to colony formation and group productivity in insects. Michener's (1964) per capita productivity paradox suggests that per capita benefits decrease as colony size increases. This observation suggests that, in many cases, individuals may be able to reproduce more efficiently alone. Yet, when considering how per capita benefits change with colony size, Michener (1964) did not account for variation in parasitoid pressure across colony phenology and whether ‘snapshot’ measures of colony productivity capture the true benefits when considered over the entire colony life cycle. For example, individuals may be better off cofounding nests if parasitism is more severe at the beginning of the reproductive season, or be more tolerant to nest joiners throughout the season if it ensures brood survival from nest invaders later in the season (e.g. Hogendoorn and Zammit 2001).

Our results provide some of the first evidence showing how resource partitioning between parasitoids may affect hosts across a univoltine reproductive season. We propose that the benefits of social nesting early in the season may only become apparent until late stages in colony development, when nests may contain only one or zero host adults, suggesting there may be a temporal dissonance between the benefits of social nesting when measured early or late in the season. Seasonally restricted “snapshot” audits of host colonies may therefore fail to reveal the true benefits of sociality. However, this presents a daunting empirical challenge: it requires that colony sizes at one point in time can be linked to colony success at later times, when colony sizes may be very different. This is not an impossible challenge, but it would require non-invasive methods for monitoring colony sizes across brood rearing phenologies and linking those to final colony success.

4.6 Statements and Declarations

The authors have no conflicts of interest to declare that are relevant to the content of this article.

4.7 Funding

This work was supported by The Holsworth Wildlife Research Endowment & The Ecological Society of Australia grant awarded to L.R.H. and in part funded by an Australian Biological Resources Study National Taxonomy Research Grant Programme grant (RF217-14) to B.A.P.

4.8 Acknowledgements

We thank Bill Wcislo and an anonymous reviewer for comments and improvements on the manuscript. Thank you to Olivia Davies, Nick Congedi and Connor Panozzo for assistance with fieldwork. This project was supported by the Australian Federal Governments Research Training Program Scholarship to L.R.H. Nests were obtained under the scientific collection permit 10008943 for Victorian National Parks.

4.9 Data availability

The analyses reported in this article can be reproduced using the dataset provided by Hearn et al. (2022).

4.10 References

Andersson M (1984) The Evolution of Eusociality. *Annual Review of Ecology and Systematics*. 15:165–189.

André E (1895) Mutillides d'Australie nouvelles ou imparfaitement connues. *Mémoires de la Société Zoologique de France*. 8:475–517.

Bergamaschi ACB, Cambra R, Melo GAR (2010) Male description and host record for *Lophomutilla corupa* Casal, 1968 (Hymenoptera: Mutillidae), with behavioural notes on mating behaviour and host nest attacks. *Journal of Natural History*. 44:2597–2607.

Brothers DJ (1989) Alternative life-history styles of mutillid wasps (Insecta: Hymenoptera). In: Bruton MN (ed) Alternative Life-History Styles of Animals. Dordrecht, Netherlands: Springer p. 279–291.

Brothers DJ, Tschuch G, Burger F (2000) Associations of mutillid wasps (Hymenoptera: Mutillidae) with eusocial insects. *Insectes Sociaux*. 47:201–211.

Bull NJ, Schwarz MP (2001) Brood insurance via protogyny: a source of female-biased sex allocation. *Proceedings of the Royal Society B: Biological Sciences*. 268:1869–1874.

Charnov EL (1982) The Theory of Sex Allocation. Princeton, New Jersey: Princeton University Press.

Danks HV (1971) Biology of some stem-nesting aculeate Hymenoptera. *Transactions of the Royal Entomological Society London*. 122:323–395.

Díaz SS, Carisio L, Manino A, Biella P, Porporato M (2021) Nesting, Sex Ratio and Natural Enemies of the Giant Resin Bee in Relation to Native Species in Europe. *Insects*. 12:545.

Farzan S, Yang LH (2018) Experimental shifts in phenology affect fitness, foraging, and parasitism in a native solitary bee. *Ecology*. 99:2187–2195.

Fox CW, Czesak ME (2000) Evolutionary Ecology of Progeny Size in Arthropods. *Annual Review of Entomology*. 45:341–369.

Frank SA (1995) Sex Allocation in Solitary Bees and Wasps. *The American Naturalist*. 146:316–323.

Gadagkar R (1990) Evolution of eusociality: the advantage of assured fitness returns. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 329:17–25.

Gerling D, Hermann H (1976) The oviposition and life cycle of *Anthrax tigrinus*, (Diptera: Bombyliidae) a parasite of carpenter bees (Hymenoptera: Xylocopidae). *Entomophaga*. 21:227–233.

Goodell K (2003) Food availability affects *Osmia pumila* (Hymenoptera: Megachilidae) foraging, reproduction, and brood parasitism. *Oecologia*. 134: 518–527.

Groulx AF, Forrest JR (2018) Nesting aggregation as a predictor of brood parasitism in mason bees (*Osmia* spp.). *Ecological Entomology*. 43:182–191.

Hardy IC (2002) Sex ratios: concepts and research methods. Cambridge, UK: Cambridge University Press.

Hearn LR, Williams KA, Stevens MI, Schwarz MP, Davies OK, Parslow BA (2019) Description and novel host records for a new species of Australian mutillid wasp (Hymenoptera: Mutillidae) from hylaeine bee nests (Hymenoptera: Colletidae). *Austral Entomology*. 58:524–532.

Hearn LR, Stevens MI, Schwarz MP, Parslow BA (2021) Parasitoids of the uniquely social colletid bee *Amphylaeus morosus* (Hymenoptera: Colletidae) in Victoria. Mem Mus Vic. 80:183–191.

Hearn LR, Parslow BP, Stevens MI, Schwarz MP (2022) Data from: Temporal dissonance between group size and its benefits requires whole-of-lifecycle measurements. *Behavioral Ecology*. Dryad, Dataset https://doi.org/10.5061/dryad.w3r2280sn

Hogendoorn K, Velthuis H (1999) Task allocation and reproductive skew in social mass provisioning carpenter bees in relation to age and size. *Insectes Sociaux*. 46:198–207.

Hogendoorn K, Zammit J (2001) Benefits of cooperative breeding through increased colony survival. *Insectes Sociaux*. 48:392–397.

Hood GR, Blankinship D, Doellman MM, Feder JL (2021) Temporal resource partitioning mitigates interspecific competition and promotes coexistence among insect parasites. *Biological Reviews*. 96:1969–1988.

IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp.

Ivanov SP (2006) The nesting of *Osmia rufa* (L.) (Hymenoptera: Megachilidae) in the Crimea: Structure and composition of nests. Entomological Review. 86:524–533.

Knerer G, Schwarz M (1976) Halictine Social Evolution: The Australian Enigma. *Science*. 194:445–448.

Lienhard A, Mirwald L, Hötzl T, Kranner I, Kastberger G (2010) Trade-off between foraging activity and infestation by nest parasites in the primitively eusocial bee *Halictus scabiosae*. *Psyche*. 2010:707501.

Lin N (1964) Increased parasitic pressure as a major factor in the evolution of social behavior in halictine bees. *Insectes Sociaux*. 11:187–192.

Lin N, Michener CD (1972) Evolution of Sociality in Insects. *The Quarterly Review of Biology*. 47:131–159.

Lundgren JG (2009) Adaptations to Pollen feeding. In: Relationships of Natural Enemies and Non-Prey Foods. Progress in Biological Control, vol 7. Dordrecht, Netherlands: Springer.

Macedo ACC, Cordeiro GD, Alves-dos-Santos I (2012) Entering behavior of *Gasteruption brachychaetum* Schrottky (Hymenoptera: Gasteruptiidae) into a nest of *Hylaeus Fabricius* (Hymenoptera: Colletidae). *Revista Brasileira de Entomologia*. 56:325–328.

Michener CD (1964) Reproductive efficiency in relation to colony size in hymenopterous societies. *Insectes Sociaux*. 11:317–341.

Michener CD (1974) The social behavior of the bees: a comparative study. Cambridge, MA: Harvard University Press.

Michener CD (1985) From solitary to eusocial: need there be a series of intervening species? *Fortschritte der Zoologie*. 31:293–305.

Mikát M, Černá K, Straka J (2016) Major benefits of guarding behavior in subsocial bees: implications for social evolution. *Ecology and Evolution*. 6:6784–6797.

Minckley RL. 1989. Host records and biological notes for two *Anthrax* species in Arizona (Diptera: Bombyliidae). *Journal of the Kansas Entomological Society*. 62:274–278.

Minckley RL, Danforth BN (2019) Sources and frequency of brood loss in solitary bees. *Apidologie*. 50:515–525.

Parslow BA (2020) Systematics and Host Associations of the Australian Gasteruption (Gasteruptiidae: Evanioidea). Ph.D. thesis, Flinders University, Adelaide.

Parslow BA, Schwarz MP, Stevens MI (2020) Review of the biology and host associations of the wasp genus *Gasteruption* (Evanioidea: Gasteruptiidae). *Zoological Journal of the Linnean Society*. 189: 1105–1122.

Polidori C, Borruso L, Boesi R, Andrietti F (2009) Segregation of temporal and spatial distribution between kleptoparasites and parasitoids of the eusocial sweat bee, *Lasioglossum malachurum* (Hymenoptera: Halictidae, Mutillidae). *Entomological Science*. 12:116–129.

Polidori C, Mendiola P, Asís JD, Tormos J, Selfa J (2010) Temporal asynchrony and spatial co-occurrence with the host: the foraging patterns of *Nemka viduata*, a parasitoid of digger wasps (Hymenoptera: Mutillidae and Crabronidae). *Journal of Ethology*. 28:353–361.

Prager SM (2014) Comparison of social and solitary nesting carpenter bees in sympatry reveals no advantage to social nesting. *Biological Journal of the Linnean Society*. 113:998–1010.

Queller DC (1989) The evolution of eusociality: Reproductive head starts of workers. *Proceedings of the National Academy of Sciences of the United States of America*. 86:3224–3226.

R Core Team (2018) *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Reeve HK, Keller L (2001) Tests of reproductive skew models in social insects. *Annual Review of Entomology*. 46:347–385.

Rehan SM, Schwarz MP, Richards MH (2011) Fitness consequences of ecological constraints and impilications for the evolution of sociality in an incipiently social bee. *Biological Journal of the Linnean Society*. 103:57–67.

Rocha-Filho LC, Melo GAR (2017) Hide and seek: is the solitary bee *Monoeca haemorrhoidalis* trying to escape from its cleptoparasite *Protosiris gigas* (Hymenoptera: Apidae: Tapinotaspidini: Osirini)? *Apidologie*. 48:262–270.

Seidelmann K (1999) The function of the vestibulum in nests of a solitary stem-nesting bee, *Osmia rufa* (L.). *Apidologie*. 30:19–29.

Seidelmann K (2006) Open-cell parasitism shapes maternal investment patterns in the Red Mason bee *Osmia rufa*. *Behavioral Ecology*. 17:839–848.

Seidelmann K, Ulbrich K, Mielenz N (2010) Conditional sex allocation in the Red Mason bee, *Osmia rufa*. *Behavioral Ecology and Sociobiology*. 64:337–347.

Shell WA, Rehan SM, Holman L (2018) The price of insurance: costs and benefits of worker production in a facultatively social bee. *Behavioral Ecology*. 29:204–211.

Smith AR, Harper C, Kapheim K, Simons M, Kingwell C, Wcislo W (2017) Effects of social organization and resource availability on brood parasitism in the facultatively social nocturnal bee *Megalopta genalis*. *Insectes Sociaux*. 65:85–93.

Smith AR, Wcislo WT, O'Donnell S (2003) Assured fitness returns favor sociality in a mass-provisioning sweat bee, *Megalopta genalis* (Hymenoptera: Halictidae). *Behavioral Ecology and Sociobiology*. 54:14–21.

Smith JA, Schwarz MP (2006) Strategic exploitation in a socially parasitic bee: a benefit in waiting? *Behavioral Ecology and Sociobiology*. 60:108–115.

Sokal RR, Rohlf FJ (1969) Biometry; the principles and practice of statistics in biological research. San Francisco: W.H. Freeman.

Soucy S (2002) Nesting Biology and socially polymorphic behaviour of the sweat bee *Halictus rubicundus* (Hymenoptera: Halictidae). *Annals of the Entomological Society of America*. 95:57–65.

Spessa A (1999) The behavioural and population ecology of an Australian native bee, *Amphylaeus morosus* Smith (Colletidae: Hylaeinae). Ph.D. thesis, Australian National University, Canberra.

Spessa A, Schwarz MP, Adams M (2000) Sociality in *Amphylaeus morosus*. *Annals of the Entomological Society of America*. 93:684–692.

Torchio PF, Tepedino VJ (1980) Sex ratio, body size and seasonality in a solitary bee, *Osmia lignaria propinqua* Cresson (Hymenoptera: Megachilidae). *Evolution*. 34:993–1003.

Wcislo WT, Minckley RL, Leschen RA, Reyes S (1994) Rates of parasitism by natural enemies of a solitary bee, *Dieunomia triangulifera* (Hymenoptera, Coleoptera and Diptera) in relation to phenologies. *Sociobiology*. 23:265–265.

Wcislo WT (1996) Parasitism rates in relation to nest site in bees and wasps (Hymenoptera: Apoidea). *Journal of Insect Behavior*. 9:643–656.

Wcislo WT (1997) Behavioral environments of sweat bees (Halictinae) in relation to variability in social organization. In: Crespi BJ, Choe JC (eds) The Evolution of Social Behaviour in Insects and Arachnids. Cambridge: Cambridge University Press. P. 316–332.

West S (2009) Sex allocation. Princeton, New Jersey: Princeton University Press.

West SA, Shuker DM, Sheldon BC (2005) Sex-ratio adjustment when relatives interact: A Test Of Constraints On Adaptation. *Evolution*. 59:1211–1228.

Yeates DK, Greathead D (1997) The evolutionary pattern of host use in the Bombyliidae (Diptera): a diverse family of parasitoid flies. *Biological Journal of the Linnean Society*. 60:149–185.

Table 1 Parasitization rates and sex ratio between early and late stages of the reproductive season. *P*-values are given for two-tailed Mann-Whitney U tests comparing the means between early and late stages of the reproductive season. In total, 263 nest cells were empty or failed due to unknown circumstances. Significant differences are in bold

		Total			**Reproductive season**						**P**
					Early			**Late**			
		***N* (total cells)**	**Mean**	**SE**	***N* (total cells)**	**Mean**	**SE**	***N* (total cells)**	**Mean**	**SE**	
Nest cells		713	4.66*	0.297	527	4.74*	0.351	186	4.45*	0.556	0.703
Parasitization rate	Total	127	0.178	0.014	71	0.135	0.015	56	0.301	0.034	**<0.001**
	Gasteruption	33	0.046	0.008	30	0.057	0.010	3	0.016	0.009	**0.023**
	Anthrax. maculatus	22	0.031	0.006	7	0.013	0.005	15	0.081	0.020	**<0.001**
	Ephutomorpha. aff. *varipes*	8	0.011	0.004	0	0	0	8	0.043	0.015	**<0.001**
	Ephutomorpha. tyla	64	0.089	0.011	34	0.065	0.011	30	0.161	0.027	**<0.001**
Sex ratio		323	0.458	0.028	250	0.508	0.032	73	0.288	0.053	**0.001**

*****Average number of cells in a nest.

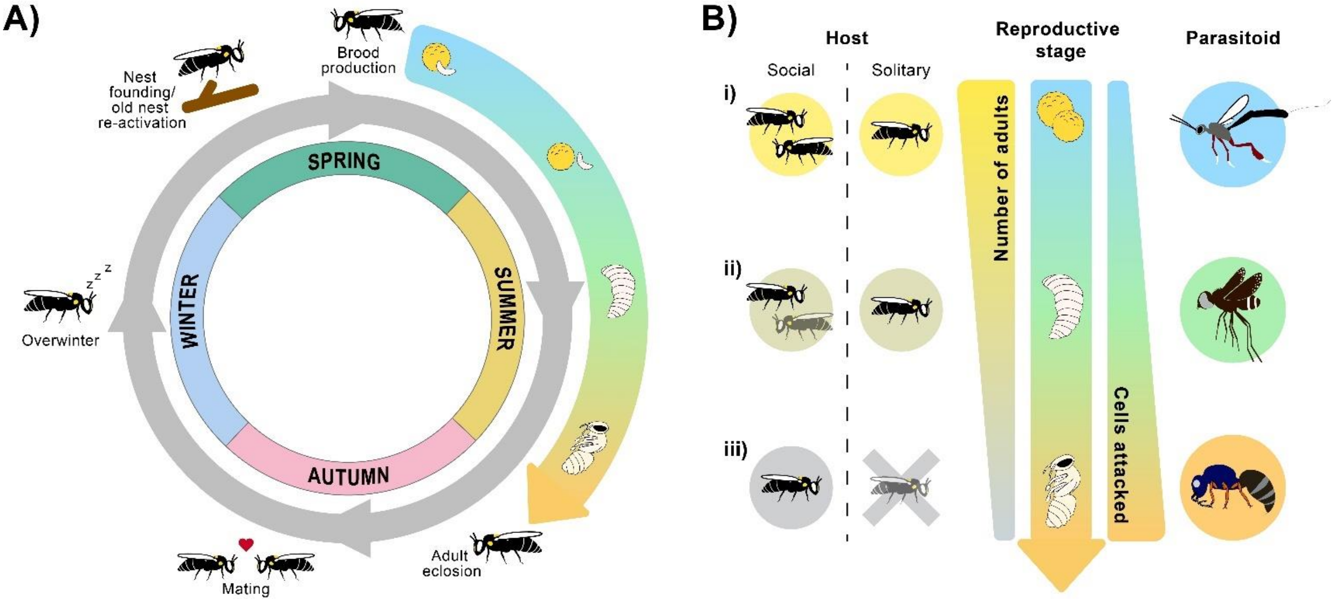

Figure 1. A) Univoltine life-cycle of *Amphylaeus morosus* showing that females mate prior to overwintering and then emerge in early spring. Adult females will then disperse to found new nests or remain in their natal nest and begin brood production. During this period from mid-spring to late summer, the potential for parasitism is high. B) Challenges facing both host and parasitoid throughout the *A. morosus* reproductive phase. i) In the early stages of the reproductive season nests are more likely to contain multiple nestmates. During this period Gasteruptiid wasps are prevalent and seek out host provisions and eggs. ii) Across the middle stages of the reproductive season there is a greater variety of host stages and potentially fewer host defenders to contend with, providing an opportunity for parasitoids such as *Anthrax maculatus* to infiltrate nests. iii) Finally, as the reproductive season progresses and brood are reaching maturity, the likelihood of having a defender remaining in the nest decreases due to abandonment or mortality. At this point, the risk of mutillid wasps extirpating full-brood nests is high. These various parasite-mediated pressures across the reproductive season create a temporal dissonance between the apparent benefits gained early in the season when parasitoid pressure is low and the benefits gained later in the season from having a defender remaining in the nest.

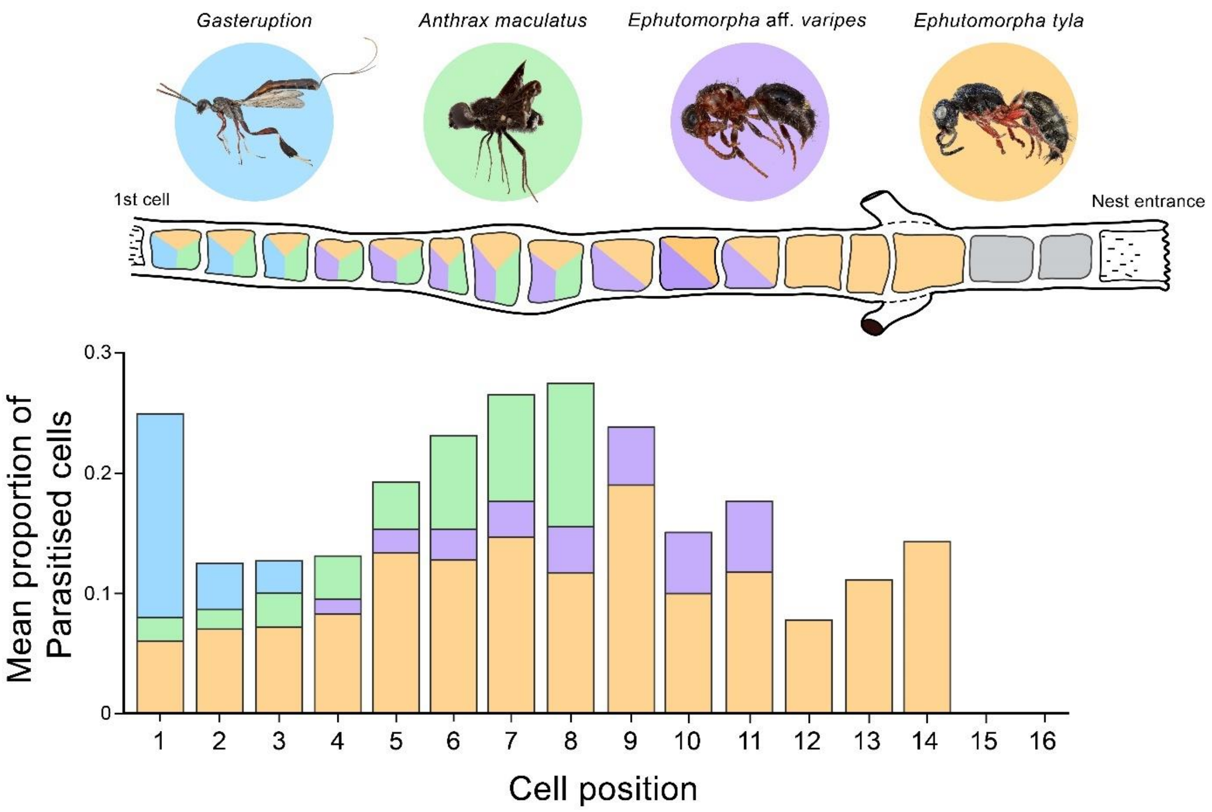

Figure 2. Representation of the linear nest structure showing the potential for a brood cell to be parasitised by a specific parasitoid species aligned with the distribution of cells parasitised by either; *Gasteruption*, *Anthrax maculatus*, *Ephutomorpha tyla* or *Ephutomorpha* aff. *varipes* across all sampling periods. No parasitization was observed in cells 15 and 16 (grey).

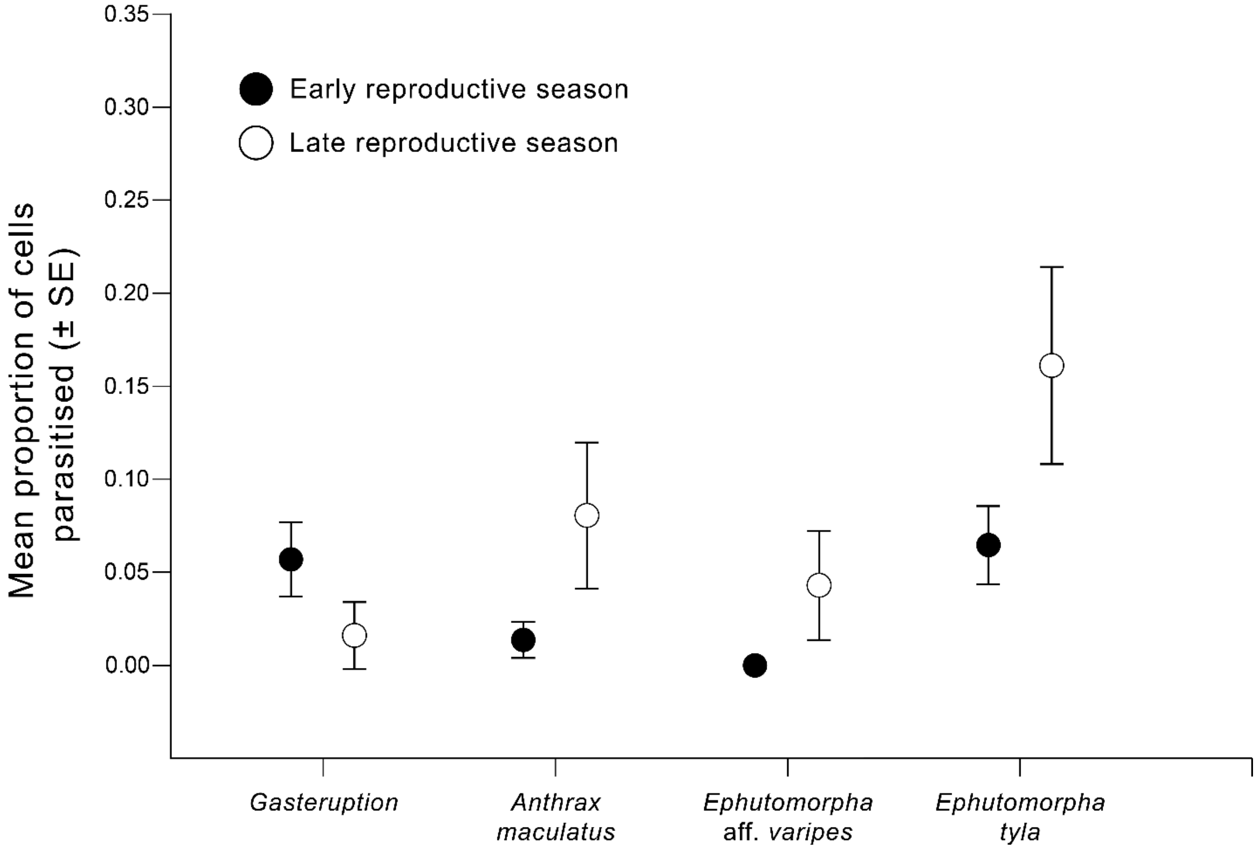

Figure 3. Mean proportion (± 1 standard error) of *Amphylaeus morosus* brood cells parasitised by each parasitoid species between early (nests collected in November and December) and late stages (nests collected January and February) of the reproductive season.

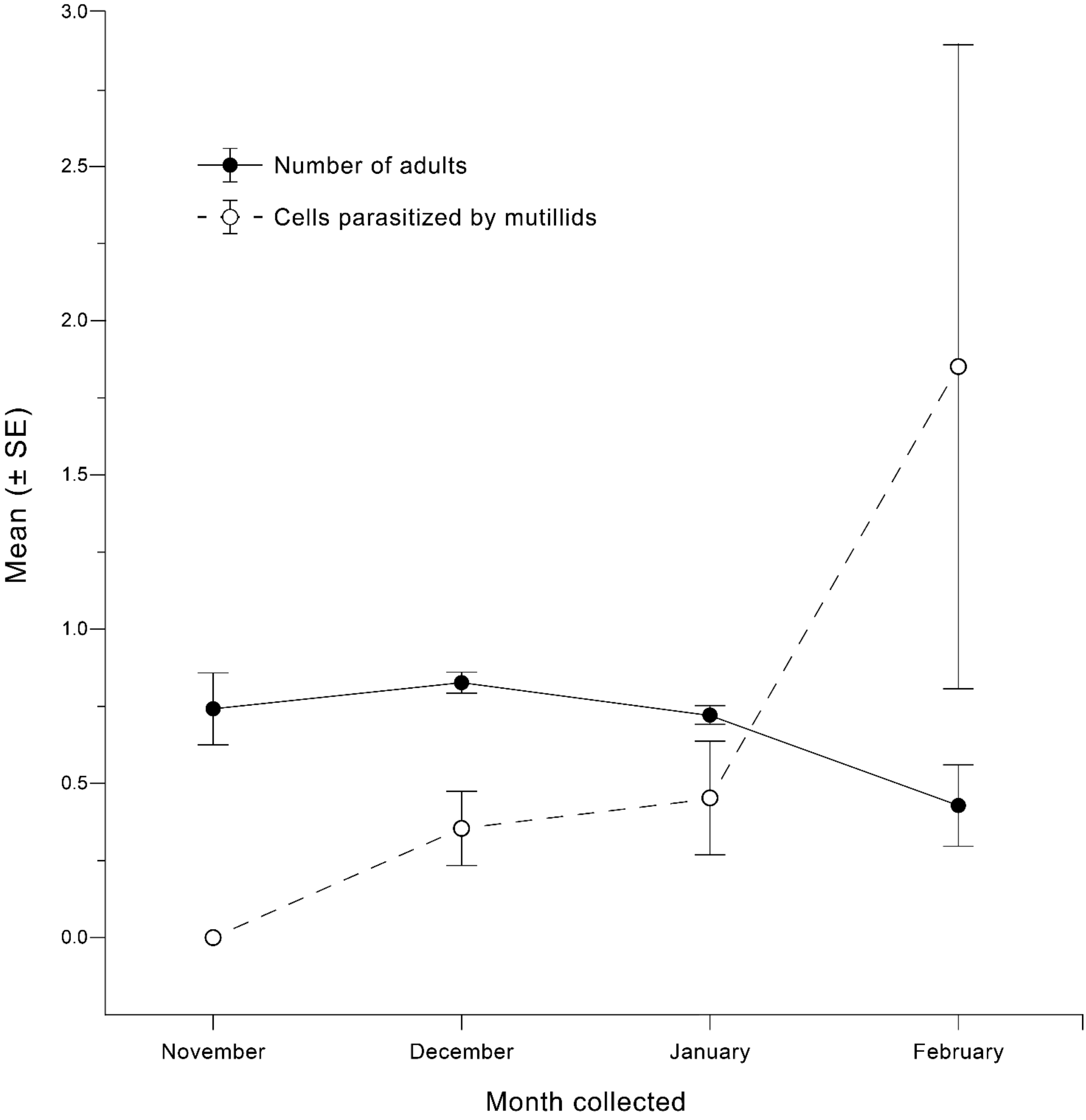

Figure 4. Mean (± 1 standard error) number of *Amphylaeus morosus* adults present in nests collected across the reproductive season and mean number of cells parasitised per nest by either mutillid species across the reproductive season.

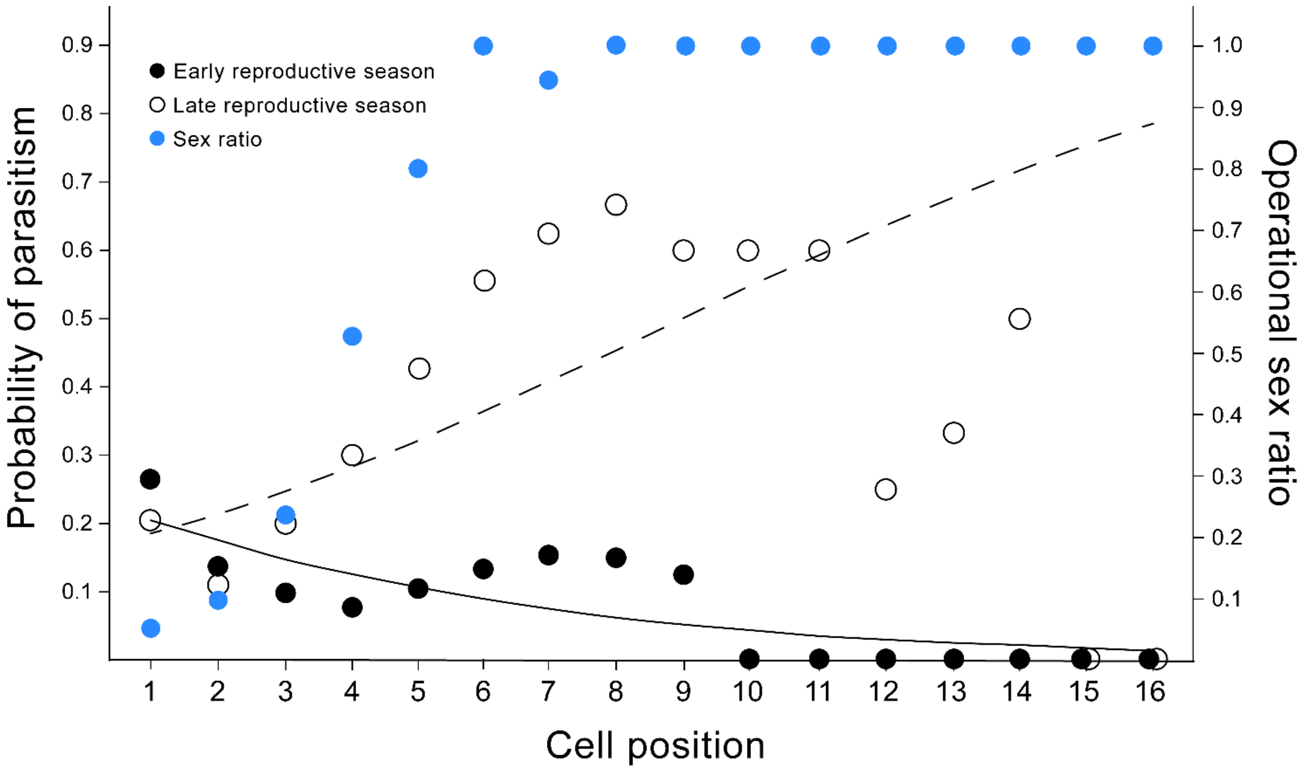

Figure 5. Risk of parasitism in relation to cell position within a nest. Observed mean proportion of parasitism (circles) at each cell position for nests collected early (nests collected in November and December) and late (nests collected January and February) in the reproductive period. Probability models (lines) calculated from a binary logistic regression equation represent the potential for parasitism at each cell position for each stage of the reproductive period (extrapolated for cell positions 15 and 16 which did not observe parasitization). Observed numerical sex ratio at each cell position shows a trend concordant to the probability of parasitism late in reproductive season. Operational sex ratios at each cell position were not incorporated into the probability models.

4.11 Supplementary material

Appendix

Table S1. Collection details for *Amphylaeus morosus* nests collected in the Dandenong Ranges, Victoria, Australia. Number of parasitised nests collected for each location and year are listed.

Nest collection						**Parasitised nests**			
Location	**GPS coordinates**	**Year**	**Date**	**Total nests**	**Total cells**	***Gasteruption***	***Anthrax maculatus***	***Ephutomorpha tyla***	***Ephutomorpha* aff. *varipes***
Gembrook/Cockatoo	37.9500° S, 145.5410° E/ 37.9350° S, 145.4920° E	2017	4 – 6 December	85	400	16	0	9	0
Sherbrooke Forest	37.8888° S, 145.3693° E	2018	6 December	6	29	0	2	0	0
Gembrook/Cockatoo	37.9500° S, 145.5410° E/ 37.9350° S, 145.4920° E	2019	21 – 22 November	27	124	7	0	0	0
Marysville/Reefton	37.5151° S, 145.7631° E/ 37.6637° S, 145.8557° E	2020	11 January	26	109	1	2	2	1
Gembrook	37.9500° S, 145.5410° E	2020	27 February	16	78	3	0	4	0

Table S2. Non-parametric Dunn-Bonferroni post-hoc comparisons of parasitized cell position for each parasitoid species. Alpha 0.05. Significant values are in bold.

	Test statistic	**Standard error**	**Standard test statistic**	**Significance**	**Adjusted significance**
Gasteruption - *A. maculatus*	-47.326	9.977	-4.744	**<0.001**	**<0.001**
Gasteruption - *E. tyla*	-48.103	7.768	-6.192	**<0.001**	**<0.001**
Gasteruption - *E.* aff. *varipes*	-75.775	14.285	-5.305	**<0.001**	**<0.001**
A. maculatus - *E. tyla*	-0.777	8.958	-0.087	0.931	1.000
A. maculatus - *E.* aff. *varipes*	-28.449	14.965	-1.901	0.057	0.344
E. tyla - *E.* aff. *varipes*	-27.672	13.593	-2.036	0.042	0.251

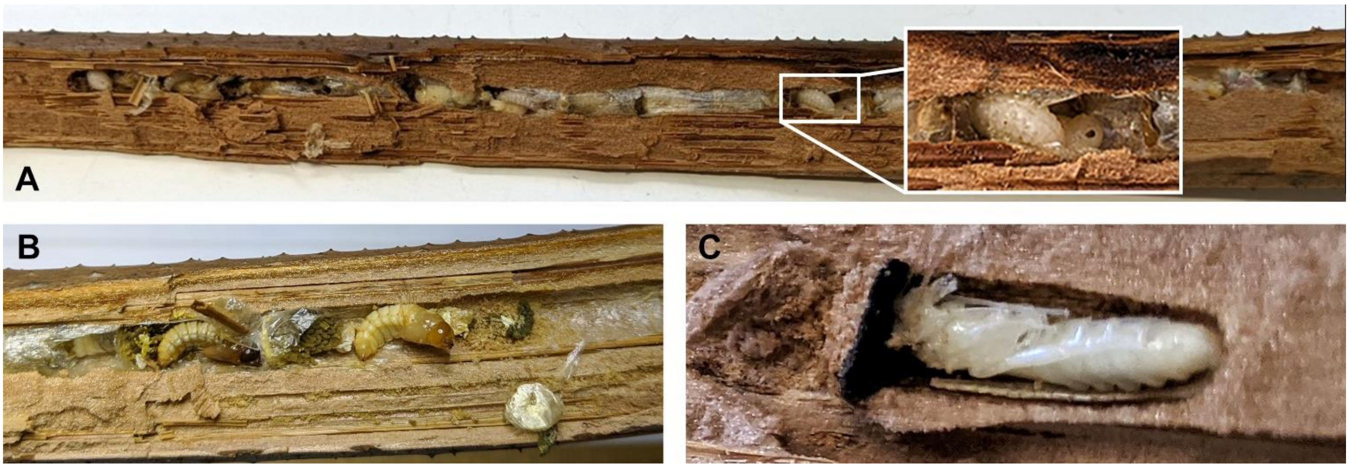

Figure S1. Parasitised *Amphylaeus morosus* nests. A) ectoparasitic *Ephutomorpha tyla* larvae feeding on *A. morosus* pupae (insert), B) *Anthrax maculatus* pupae developing in broken host brood cells and, C) *Gasteruption* pupa developing in the first cell, separated from the rest of the nest by a dark hard partition.

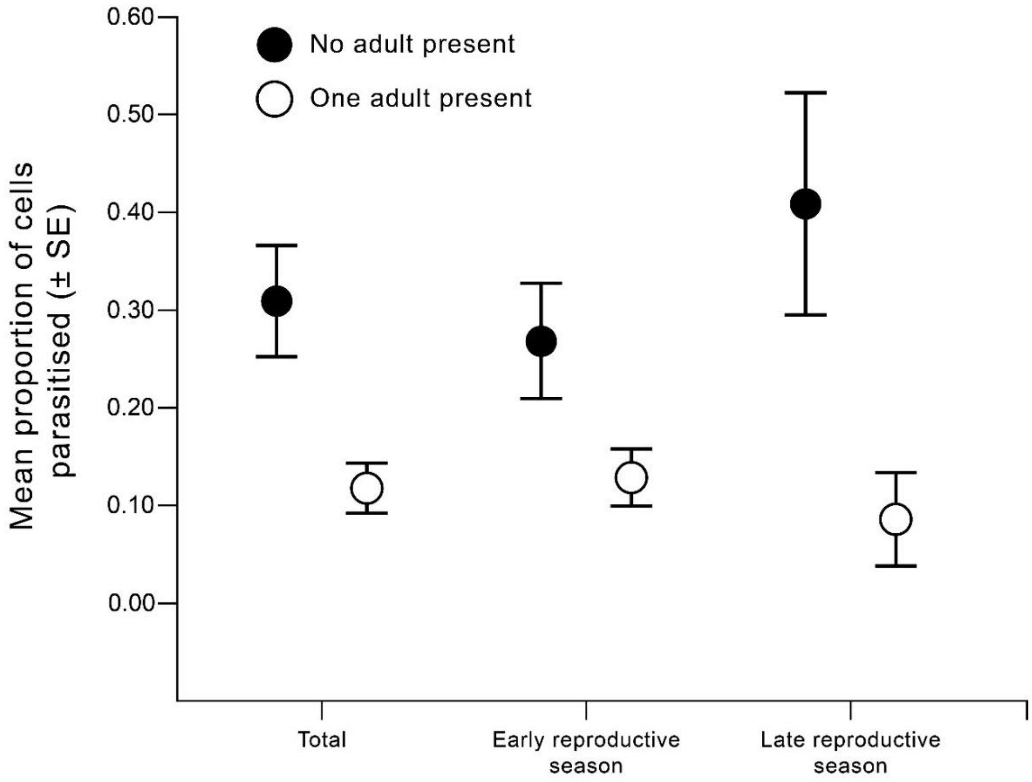

Figure S2. Mean proportion (± 1 standard error) of parasitised cells in nests containing an adult female and nests with no adult female.

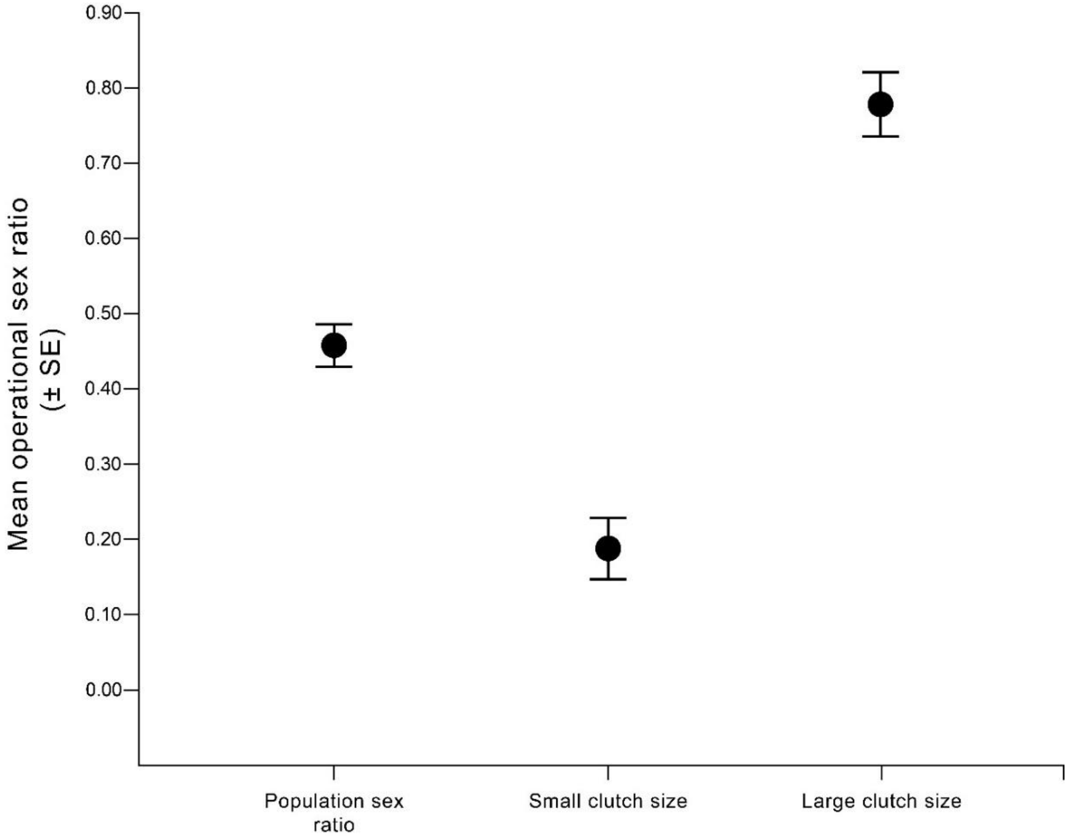

Figure S3. Comparison of mean (± 1 standard error) sex ratios, presented as male:female, for nests with small clutches (less than six offspring), nests with large clutches (six or more offspring) and the population operational sex ratio.

Chapter 5 – Extreme reproductive skew at the dawn of sociality is consistent with inclusive fitness theory but problematic for routes to eusociality

This chapter has been published in Proceedings of the Royal Society B

https://doi.org/10.1098/rspb.2022.0652

Lucas R. Hearn[1*], Olivia K. Davies[1] and Michael P. Schwarz[1]

[1] *College of Science and Engineering, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia.*

*Corresponding author: Lucashearn7@gmail.com

5.1 Abstract

To understand the earliest stages of social evolution we need to identify species that are undergoing the initial steps into sociality. *Amphylaeus morosus* is the only unambiguously known social species in the bee family Colletidae and represents an independent origin of sociality within the Apoidea. This allows us to investigate the selective factors promoting the transition from solitary to social nesting. Using genome-wide SNP genotyping, we infer robust pedigree relationships to identify maternity of brood and intracolony relatedness for colonies at the end of the reproductive season. We show that *A. morosus* forms both matrifilial and full-sibling colonies, both involving complete or almost complete monopolization over reproduction. In social colonies, the reproductive primary was also the primary forager with the secondary female remaining in the nest, presumably as a guard. Social nesting provided significant protection against parasitism and increased brood survivorship in general. We show that secondary females gain large indirect fitness benefits from defensive outcomes, enough to satisfy the conditions of inclusive fitness theory, despite an over-production of males in social colonies. These results suggest an avenue to sociality that involves high relatedness and, very surprisingly, extreme reproductive skew in its earliest stages and raises important questions about the evolutionary steps in pathways to eusociality.

Key words: Social evolution, Inclusive fitness theory, Eusociality, Reproductive skew, Hymenoptera

5.2 Significance

The evolutionary transition from solitary living to eusociality is thought to have evolved via a series of progressive stages that allow complex behaviours, such as reproductive skew and morphological castes, to gradually develop. Using a species that is taking its initial steps into social living, we show that social behaviour in the Australian native bee, *Amphylaeus morosus*, consists of high inclusive fitness benefits and extreme reproductive skew in the absence of morphological differentiation between social nestmates. We propose that extreme reproductive skew can arise in early social evolution in ways that are consistent with Hamilton’s inclusive fitness theory. But this very early appearance of near-or-total altruist sterility changes the fitness landscapes that are usually assumed in traditionally proposed routes to eusociality.

5.3 Introduction

The evolution from solitary living to complex sociality involving sterile worker castes represents a major evolutionary transition and continues to generate debate surrounding the conditions under which eusociality can evolve (Szathmáry and Smith 1995). It is generally thought that the evolution from solitary to eusociality has arisen through a series progressive evolutionary steps referred to as a 'social ladder' (Linksvayer and Johnson 2019; Rehan and Toth 2015; Wilson 1971). The underlying assumption of this thinking is that extant species presenting similar forms of eusocial behaviour have passed through similar 'rungs on a social ladder' and have undergone gradual behavioural and genetic changes overtime (Linksvayer and Johnson 2019; Rehan and Toth 2015, but see Kapheim et al. 2015). Any significant social jumps that are able to hurdle intermediate 'rungs on the social ladder' are considered unlikely, especially jumps from solitary nesting to complex sociality (Hunt 2007, but see Michener 1985). A key feature that might promote such a rapid rise to eusociality is the differentiation between totipotent nestmates at an early stage of social evolution in combination with evolutionary drivers that might offset an individual's reproductive sacrifice (Gadagkar et al. 1990).

Eusociality has received particular attention in social evolution studies because it typically involves extreme reproductive skew, entailing morphological castes and effective worker sterility (Bourke 1988). It has even been suggested that worker sterility may release species from the constraints of gene-level inclusive fitness models (Nowak et al. 2010, but see Abbot et al. 2011). When trying to understand the evolution of eusociality, a major and on-going question could be simplistically framed as a 'chicken-or-egg' problem: did worker castes arise before helper sterility, or did effective sterility allow worker morphologies to evolve later on? That issue cannot be readily addressed using taxa such as ants, termites or honeybees because they do not contain extant species that might approximate conditions when sociality was first evolving (Jones et al. 2017; Linksvayer and Johnson 2019). Instead, we need to examine species where sociality has only recently evolved and where altruistic behaviour is not obligate.

Many previous attempts to elucidate the evolutionary transition from primitive to complex forms of sociality have frequently used species that were supposedly in the early steps of acquiring social traits but have turned out to involve species that are derived from ancestors where social behaviour was more complex, and so represent 'reversions' to seemingly less complex behaviour (Danforth 2002; Gadagkar 1990; Leadbeater et al. 2011; Richards et al. 2003). Consequently, these species may not provide strong insights into the very earliest stages of social evolution. To understand the earliest steps into sociality we need to identify species that are still in early phases of social evolution.

The Australian native bee *Amphylaeus morosus* (Hymenoptera: Colletidae) is the only known social bee in the hyper-diverse Colletidae bee family. Social behaviour in *A. morosus* is facultative, with solitary and social nests occurring within the same population, sometimes within centimetres of each other (Fig. 1C). Social nests are infrequent and generally contain two nestmates, though they can rarely contain up to three individuals, but always re-using natal nests from the previous year (Spessa et al. 2000). In the montane populations of south-eastern Australia, *A. morosus* is strictly univoltine; however, Houston (1969) suggested the potential for more than one generation to be produced in the subtropical populations of southern Queensland, Australia. Females mate before overwintering and may either disperse and establish new nests in early spring or remain in their natal nests which are then re-used (Fig. S3). Brood cells are laid in a linear sequence, with mothers ovipositing onto semi-liquid provisions before sealing cells (Fig. 1B). In the Dandenong Ranges, Victoria, Australia, *A. morosus* is attacked by at least eight different parasitoid species with staggered windows of attack driven by host resource utilisation (Hearn et al. 2021). These parasitoids provide severe ecological pressures throughout the reproductive season and have been shown to dramatically increase the benefits of having at least one defender in the nest at the end of the reproductive season (Hearn et al. 2022).

Using *A. morosus* we demonstrate extreme reproductive skew at the dawn of sociality in the only known social species in the bee family Colletidae. We show that even in this very early stage of social evolution extreme reproductive skew can evolve and be consistent with inclusive fitness theory. This kind of early extreme skew suggests an evolutionary landscape that is very different from previously hypothesized routes to eusociality.

5.4 Methods

Nest collection

Nests of *Amphylaeus morosus* were collected from the Dandenong Ranges, Victoria, Australia. Nests were sampled from seven separate collections during austral spring and summer across five years from 2017–2021. Within the Dandenong Ranges, *A. morosus* nests in dead abscised fronds of the rough fern tree, *Cyathea australis*. Nests were opened longitudinally up to the first brood cell so that mothers and additional contents preceding the first cell could be removed. Immature brood were left in the nest to develop to adult eclosion prior to extraction, at which point they were sexed. Upon nest opening, the contents including number of brood, parasitised brood and surviving brood were recorded. Brood and their presumptive mothers/alloparents were removed from the nest and placed in 99% ethanol for genetic sequencing.

Genotyping

Single nucleotide polymorphisms (SNPs) for 197 brood and 31 potential mothers collected from 20 nests over three collection periods (Table S1) were assayed using high-throughput microarray sequencing following the methods described in (Jaccoud et al. 2001) using the DArTseq™ (Diversity Arrays Technology sequencing) proprietary platform, completed by Diversity Arrays Technology Pty. Ltd. (Canberra, ACT, Australia). DNA was extracted from the head or thorax of each specimen to optimise DNA quality and minimize potential DNA contamination from gut microbiomes in metasomal tissue. Preliminary sequences revealed no differences in the sequencing depth between head and thoracic tissues.

Loci quality filtering

A total of 17,194 SNP loci were called for the 228 assayed individuals of *A. morosus*. Quality filtering of SNP markers was performed using the dartR package version 1.9.9.1 (Gruber et al. 2018), implemented in R version 4.0.4 (R Development Core Team 2020). SNPs were filtered for minor allele frequencies below 5%, SNPs that share a sequence tag, monomorphic loci, loci with a repeatability less than 99%, and loci with a call rate less than 99%. Loci showing apparent linkage disequilibrium (LD) were filtered using the R package SNPRelate version 1.28 (Zheng et al. 2012) at an r^2 threshold of 0.8, resulting in the retention of 950 loci.

Measure of allelic diversity were conducted using all adult females from each nest and four additional adults independent from the samples collected (n = 31). The population inbreeding coefficient, F_{IS} and departures from Hardy-Weinberg equilibrium were calculated in the R package SNPRelate (*SI Appendix*). To avoid any confounding influences from genetic correlations among related individuals, calculations of population allele frequencies were weighted by the inverse of colony size.

Pedigree assignments and relatedness estimates

To explore patterns in parentage, pedigree relationships were reconstructed for the brood and potential mothers of all genotyped individuals using the program COLONY version 2.0.6.6. (Wang 2004). A maximum likelihood approach was used to infer putative pedigrees based on population allele frequency. This approach accounts for genotyping errors, where the genotyping error rate was set to 0.001. Details of the parameters used for these analyses are given in *SI Appendix*.

Pairwise relatedness and mean intracolony relatedness for each nest group were estimated using the program KINGROUP V2 (Konovalov et al. 2004). The putative pedigrees from COLONY (based on highest log probability) were used to partition individuals into groups. The pairwise relatedness between both adult females-to-offspring and offspring-to-offspring in a given colony was calculated using the relatedness estimator in KINGROUP V2 (Queller and Goodnight 1989) based on population allele frequencies where colonies, rather than individuals, were given equal weight.

Measuring reproductive skew

Reproductive skew was calculated using the software SKEW CALCULATOR 2003 (Nonacs 2003) for all genotyped social nests where maternity of brood could be confidently evaluated. We used the binomial skew index (*B*) (Nonacs 2000) which tests within each group if the observed variance in reproduction significantly differs from the expected variance if all group members have an equal probability to reproduce (*SI Appendix*).

Dissection data

Adult females from all sequenced nests (n = 21 females) and adult females from an additional three solitary (n = 3 females) and three social nests (n = 6 females) were placed in 70% ethanol for 24 hours prior to dissection. Individuals were dissected under a Leica MS5 stereomicroscope and measurements were taken for wing length, wing wear, ovary size and mated status. To avoid problems with some occasionally very worn wing margins, wing length was measured as the distance from the axillary sclerites to the apex of the marginal cell on the forewing. In *A. morosus* wing length was found to have a linear relationship with body weight and intertegular distance of brood that had just reached adulthood and was therefore used as a proxy for body size (Fig S4). Wing wear was assessed as the number of nicks in the distal forewing margins and was used as a proxy of individual age and foraging activity (Joyce and Schwarz 2006). Ovary size was measured as the arithmetic mean length of the three largest oocytes. Mated status was determined by observing the presence of sperm in the spermatheca. Monte Carlo resampling procedures were used to determine whether morphological hierarchies existed within social nests (*SI Appendix*).

Fitness estimation

Indirect fitness estimates were calculated by multiplying the expected pairwise relatedness between social nestmates (i.e. mother-daughter = 0.5; full-sisters = 0.75) by the benefit of social nesting, which was estimated as the difference between the mean number of offspring that survived to adulthood in social nests for matrifilial and full-sibling strategies and the mean number of offspring that survived to adulthood in solitary nests.

Statistical analyses

All statistical analyses were performed in R version 4.0.4 (R Development Core Team 2020). We used Mann-Whitney and Kruskal-Wallis tests to assess differences in total brood output, surviving brood, and parasitisation rates between solitary and social nests. Colonies were grouped based on the number of adult females present at the time of collection. For some analyses, we classified colonies where the social dynamics were ambiguous at the time of collection as follows — nests with no adult females at the time of collection ($N = 86$ colonies) as solitary and genome-inferred multi-female nests (colonies where one mother had abandoned the nest prior to collection; $N = 3$ colonies) as social nests. Unless otherwise stated, values are presented as mean ± SE.

5.5 Results

Pedigree assignments and intracolony relatedness

Amphylaeus morosus rears a single brood per year, with egg-laying commencing in spring and adults emerging in mid-to-late summer. Colony sizes during brood rearing were very small, with nests containing a maximum of two adult females. The mean pairwise relatedness, based on 950 genome-wide SNP loci, for adult nestmates in social colonies was $r = 0.589 \pm 0.075$ ($N = 10$). Of 20 genotyped colonies, comprising both solitary and social nests, SNP-based pedigree assignments revealed eighteen that contained a female who could be confidently assigned as mother to all the offspring in the nest. In two nests the only adult present had not produced offspring, and in one of these the offspring were entirely unrelated to the adult female present in the nest at the time of collection.

Maternity analyses and intracolony relatedness revealed the existence of both matrifilial and full-sibling colonies, both containing one ‘primary’ female who produced all or most brood, and another ‘secondary’ female who produced zero or only one brood (Table 1). In matrifilial colonies, the adult daughter was the secondary and exhibited no observable damage to their wings, whereas mothers had highly worn wing margins, consistent with extended age and/or activity (Table S6). A similar pattern was seen for full-sibling colonies: full monopolisation over reproduction was found in five full-sibling colonies and in only one nest was a brood cell laid by the secondary female (a single male offspring in last cell of the nest) (Fig. 1A). In all social nests, the reproductive primary was inferred to be the primary forager based on wing wear. Reproductive skew was not significantly different between matrifilial colonies and full-sibling colonies (*t* test: $t_7 = 1.07$, $P = 0.319$) and had no significant relationship with pairwise relatedness between adult nestmates ($F_{1,7} = 1.465$, $P = 0.265$).

Offspring numerical sex ratio was significantly male biased in all sequenced social nests when offspring were pooled across nests (pooled numerical ratio = 0.827, $\chi^2_1 = 48.89$, $P < 0.001$; Table 1), in contrast, the sex ratio for solitary nests did not differ from a 1:1 ratio (pooled numerical ratio = 0.517, $\chi^2_1 = 0.892$, $P = 0.345$). When the numerical sex ratio was calculated for each social colony, the mean numerical colony ratio was also male biased and significantly different from 0.5 (mean numerical ratio r = 0.823, one sample $t_9 = 12.81$, $P < 0.001$). Sex ratio was significantly influenced by the relatedness between adult nestmates ($F_{1,7} = 19.42$, $P = 0.003$).

Nestmate morphological hierarchies

All of the dissected adult females were mated ($N = 21$ colonies, $n = 30$ females). To explore if any morphology-based hierarchies occurred between social nestmates, we compared individuals in two-female social colonies for differences in ovary size, body size and wing wear. Independent-samples t-tests determined there was no statistical difference in mean ovary size and mean body size between adult female nestmates ($t_{16} = 1.96$, $P = 0.067$; $t_{16} = 1.73$, $P = 0.103$), but mean wing wear was significantly different ($t_{16} = 3.49$, $P = 0.003$). To further untangle these differences, we used Monte Carlo techniques to simulate social nests which confirmed these patterns (*SI Appendix*).

Benefits of social nesting

Social nests ($N = 13$ colonies, 4.3% of sampled nests) were rare throughout the Dandenong Ranges compared to solitary nests ($N = 289$, 95.7% of sampled nests) and only contained up to two adult females at the times of collection. However, genetic sequencing of colonies revealed three ‘hidden’ social nests where a mother could be inferred but was no longer present, making it likely that social colonies are more common than our sampling effort was able to capture. On average, social nests were more productive than solitary nests in regard to reproductive output (solitary: 5.11 ± 0.22, $N = 289$; social: 12.31 ± 0.56, $N = 13$; Mann-Whitney: U = 317, $P < 0.001$; Fig 2A). Nests with one female present at the time of collection contained a similar number of provisioned brood cells to nests with no adult female (one adult female: 5.29 ± 0.27, $N = 203$; no adult female: 4.56 ± 0.38, $N = 86$; Mann-Whitney: U = 7669.5, $P = 0.068$). However, nests with one adult female had significantly more offspring survive to adulthood (one adult female: 3.41 ± 0.26, $N = 203$; no adult female: 1.24 ± 0.27, $N = 86$; Mann-Whitney: U = 4666.5, $P < 0.001$; Fig 2B).

Indirect fitness for secondaries

Indirect fitness was estimated for social secondaries in matrifilial and full-sibling colonies (*SI Appendix*). On average, secondary females in full-sibling colonies had slightly higher mean indirect fitness (mean = 5.54 ± 0.995) compared to secondary females in matrifilial colonies but no statistical difference was detected (mean = 4.45 ± 0.441; Mann-Whitney: U = 7.00, $P = 0.599$; Fig 2C). Inclusive fitness was estimated as the combined direct and indirect fitness gain for primaries and secondaries respectively (Table 2).

The genetic investment of secondary females to the primary female's offspring was calculated for matrifilial and full-sibling reproductive strategies as follows: matrifilial: $n_{\text{(female offspring)}} \times 0.75 + n_{\text{(male offspring)}} \times 0.25$; full-sibling: $(n_{\text{(female offspring)}} + n_{\text{(male offspring)}}) \times 0.375$. The genetic investment of secondary females in full-sibling colonies (mean = 3.69 ± 0.51) was not significantly higher than secondary females in matrifilial colonies (mean = 3.42 ± 0.22; Mann-Whitney: U = 8.00, $P = 0.795$; Fig 2D).

5.6 Discussion

Our use of genome-wide SNPs indicated extreme reproductive skew in both matrifilial and full-sibling colonies in a species that was previously thought to be egalitarian and consisting of only weakly related individuals (Spessa et al. 2000). Importantly, we find that this extreme skew is consistent with Hamilton's Rule (Hamilton 1964*a,b*), yet hierarchies based on size or morphology are absent. On the other hand, we did find one nest containing an adult female that was unrelated to any of the brood, suggesting a low level of drifting behaviour (Nonacs 2017; Ulrich et al. 2009). Our data indicates that social nesting in *Amphylaeus morosus* is uncommon (<5% of the nests sampled), and this raises questions as to the benefits of nest sharing. In *A. morosus*, the indirect fitness benefits for social secondaries exceeded the benefits of reproducing alone (Table 2) and demonstrated a significant advantage to social nesting. *Amphylaeus morosus* is host to a diverse suite of parasitoids that attack at different stages throughout the reproductive season, including severe risk of mortality from mutillid wasps at the end of the season when nests contain full broods (Hearn et al. 2022). This parasite pressure is likely to be a key driver promoting cooperative nest defence in this species (Lin and Michener 1972).

Intracolony relatedness for *A. morosus* was previously estimated at *r*=0.26 (Spessa et al. 2000) and this discrepancy between our higher relatedness estimate is likely a factor of the resolution of the available technology (allozymes) used at the time. Matrifilial associations of adults during brood rearing were not considered as possibilities in that earlier study of *A. morosus* (Spessa et al. 2000), but our pairwise relatedness estimates combined with wing wear patterns make this colony composition apparent in some nests (*SI Appendix*), indicating that some individuals in this species can survive across consecutive years. Extended longevity of adults, allowing adult mother-daughter associations, is widely regarded as a prerequisite of eusociality (Alexander 1974; Andersson 1984; Crespi and Yanega 1995; Wilson 1971). At the same time, we did not find that reproductive skew differed between matrifilial and full-sibling colonies. This is important because it indicates that extreme skew can arise regardless of the potential for this to subsequently evolve into queen-worker systems that are based on mother-daughter relationships. Additionally, we found no evidence of ovarian development suppression in the secondary females, which does not support the de-coupling model for the evolution of morphologically identifiable castes in the early stages of social development (West-Eberhard 1996). This prompts the question of whether matrifilial associations and distinct reproductive phenotypes really are pre-conditions for the evolution of eusociality; instead, the existence of extreme skew could create selection for increased adult longevity that could exploit already-present queen-like roles. We note that under some definitions (Dew et al. 2016), some colonies of *A. morosus* could be regarded as eusocial based on reproductive monopolisation by mothers in matrifilial colonies.

Using allodapine bees as an exemplar life-cycle, Michener (1985) proposed that eusociality could rapidly evolve from subsocial colonies if first-emerging daughters simply switch from producing their own offspring to rearing their younger siblings. However, subsequent studies have shown that allodapine and xylocopine species with high levels of reproductive skew can comprise matrifilial, full-sibling, as well as less related compositions of adult females, as seen in the carpenter bee *Xylocopa sulcatipes*, where initially subordinate helpers may inherit the nest and become reproductive (Harradine et al. 2012; Stark 1992). Matrifilial and full-sibling colonies have often been associated with the subsocial and semi-social routes to eusociality, respectively, yet those hypothetical pathways operate under distinctly separate evolutionary dynamics (da Silva 2021; Kocher and Paxton 2014; Lin and Michener 1972; Michener 1985). Subsociality could potentially favour worker-like daughters because of high full-sister relatedness in haplodiploids, whereas the semisocial route is thought to be more strongly driven by mutualistic factors, with worker-like behaviour evolving once colonial life has become established (Lin and Michener 1972). In *A.*

morosus social nests, sex ratios were heavily male-biased resulting in no significant difference in either the inclusive fitness benefits or the genetic investment of secondary females when comparing matrifilial and full-sibling colonies. In other words, the potential for helper daughters to exploit high sister-sister relatedness was negated by mothers producing more sons than daughters.

The ability for workers to assess relatedness asymmetries and augment their indirect fitness by skewing sex allocation towards sisters has been reported for some eusocial Hymenoptera (Meunier et al. 2008) but it is unclear if such biased alloparental care could promote origins of eusociality rather than simply be a response by already-existing worker castes (Bourke 1988; Gardner et al. 2012). However, such worker-controlled sex ratios are not possible in *A. morosus*, given that killing a mother's male egg would result in a provisioned cell without any brood, or else the secondary female would have to replace a primary's egg with her own and neither of these outcomes were detected in our sequenced colonies. The over-production of male brood may also limit the extent of social nesting within the population: if the presence of a guard increases the number of male brood, the increased number of males entering the population will lower their mean reproductive success, leading to diminishing indirect fitness returns for secondaries as they become more frequent in a population.

The potential for secondary females to occasionally survive into a second year of brood rearing and then assume reproductive dominance raises the question of reproductive queuing. Our estimates of inclusive fitness for secondary females in *A. morosus* did not explore this possibility, but reproductive queuing that entails alternation between initial indirect fitness gains with subsequent direct fitness has been shown for the large carpenter bee, *Xylocopa sulcatipes* (Stark 1992), and has been posited for multiple allodapine bee species where it can eventually result in the evolution of permanent worker castes (Schwarz et al. 2011). The costs of dispersal and social contests between nestmates may also be important factors determining why secondaries choose to stay in a social nest and relinquish direct reproduction (Heinze 2010). While nesting sites do not appear to be limiting in the Dandenong Ranges (Spessa et al. 2000) the costs of constructing new nests has not been estimated, though Spessa et al. (Spessa et al. 2000) showed that brood provisioning begins earlier in social re-used nests than newly constructed ones.

High intracolony relatedness and reproductive altruism are characteristics underlying the most advanced forms eusociality and while these characteristics are sometimes present in small totipotent societies of extant halictine, ceratinine, allodapine bees and polistine wasps, those are not truly representative of the earliest stages of social evolution as they arise from lineages with very long histories of prior social behaviour (Linksvayer and Johnson 2019; Michener 1985; Rehan et al. 2012). Using *A. morosus*, a species in very early stages of social evolution, we have shown that very early forms of sociality can entail extreme reproductive skew in the absence of morphological castes, but this altruism is consistent with Hamilton's inclusive fitness theory. These two findings suggest that effective worker sterility can arise very early on in social evolution (an idea also raised by Michener (1985) and West-Eberhard (1996)), yet it remains a feature that is not accounted for in most proposed pathways to eusociality. Furthermore, the indirect fitness gains from altruist sterility are not significantly different between matrifilial and full-sibling colonies. When combined, these features do not fit neatly into proposed early steps in any of the hypothesized routes to eusociality and, we argue, those routes need to be re-evaluated.

5.7 Acknowledgements

We thank Tyla Cousins and Georgia Macaulay for assistance with fieldwork and Rebecca Dew for help with R scripts. This project was supported by the Australian Federal Governments Research Training Program Scholarship and the Holsworth Wildlife Research Endowment & The Ecological Society of Australia grants awarded to L.R.H. and O.K.D. Nests were obtained under the scientific collection permit 10008943 for Victorian National Parks.

5.8 Author contributions

L.R.H., O.K.D. and M.P.S. designed the research, L.R.H. and O.K.D collected the data, L.R.H and M.P.S. analyzed the data, L.R.H., O.K.D. and M.P.S. wrote the manuscript. All authors read a final version of the manuscript.

5.9 Data availability

The analyses reported in this article can be reproduced using the dataset provided by Hearn et al. (50).

5.10 References

Abbot P et al. (2011) Inclusive fitness theory and eusociality. *Nature*. 471:E1–4; author reply E9-10.

Alexander RD (1974) The evolution of social behaviour. *Annual Review of Ecology and Systematics*. 5:325–383.

Andersson M (1984) The Evolution of Eusociality. *Annual Review of Ecology and Systematics*. 15:165–189.

Bourke AFG (1988) Worker Reproduction in the Higher Eusocial Hymenoptera. *The Quarterly Review of Biology*. 63:291–311.

Crespi BJ, Yanega D (1995) The definition of eusociality. *Behavioral Ecology*. 6:109–115.

Dew RM, Tierney SM, Schwarz MP (2016) Social evolution and casteless societies: needs for new terminology and a new evolutionary focus. *Insectes Sociaux*. 63:5–14.

da Silva J (2021) Life History and the Transitions to Eusociality in the Hymenoptera. *Frontiers in Ecology and Evolution*. 9.

Danforth BN (2002) Evolution of sociality in a primitively eusocial lineage of bees. *Proceedings of the National Academy of Sciences*. 99:286–290.

Gadagkar R (1990) Origin and evolution of eusociality: a perspective from studying primitively eusocial wasps. *Journal of Genetics*. 69:113–125.

Gadagkar R, Hamilton WD, Bone Q (1990) Evolution of eusociality: the advantage of assured fitness returns. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 329(1252):17–25.

Gardner A, Alpedrinha J, West SA (2012) Haplodiploidy and the evolution of eusociality: split sex ratios. *The American Naturalist*. 179:240–256.

Gruber B, Unmack PJ, Berry OF, Georges A (2018) dartr: An r package to facilitate analysis of SNP data generated from reduced representation genome sequencing. *Molecular Ecology Resources*. 18:691–699.

Hamilton WD (1964a) The genetical evolution of social behvaiour I. *Journal of Theoretical Biology*. 7:1–16.

Hamilton WD (1964b) The genetical evolution of social behvaiour II. *Journal of Theoretical Biology*. 7:17–52.

Harradine SL, Gardner MG, Schwarz MP (2012) Kinship in a social bee mediates ovarian differentiation and has implications for reproductive skew theories. *Animal Behaviour*. 84:611–618.

Hearn LR, Davies OK, Schwarz MP (2022), Extreme reproductive skew at the dawn of sociality is consistent with inclusive fitness theory but problematic for routes to eusociality. Dryad, Dataset https://doi.org/10.5061/dryad.wdbrv15r5.

Hearn LR, Parslow BA, Stevens MI, Schwarz MP (2022) Temporal dissonance between group size and its benefits requires whole-of-lifecycle measurements. *Behavioural Ecology*. 33:606–614.

Hearn LR, Stevens MI, Schwarz MP, Parslow BA (2021) Parasitoids of the uniquely social colletid bee *Amphylaeus Morosus* (Hymenoptera: Colletidae) in Victoria. *Memoirs of Museum Victoria*. 80:183–191.

Heinze J (2010) Conflict and conflict resolution in social insects. In: Kappeler P (ed) Animal Behaviour: Evolution and Mechanisms. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 151–178.

Houston T (1969) The systematics and biology of Australian Hylaeine bees (Hymenoptera: Colletidae). Ph.D. thesis, University of Queensland, Hertfordshire, St Lucia.

Hunt JH (2007) The evolution of social wasps. Oxford University Press.

Jaccoud D, Peng K, Feinstein D, Kilian A (2001) Diversity arrays: a solid state technology for sequence information independent genotyping. *Nucleic Acids Research*. 29:e25–e25.

Jones BM, Kingwell CJ, Wcislo WT, Robinson GE (2017) Caste-biased gene expression in a facultatively eusocial bee suggests a role for genetic accommodation in the evolution of eusociality. *Proceedings of the Royal Society B: Biological Sciences*. 284:20162228.

Joyce NC, Schwarz MP (2006) Sociality in the Australian allodapine bee *Brevineura elongata*: small colony sizes despite large benefits to group living. *Journal of Insect Behavior*. 19:45–61.

Kapheim KM et al. (2015) Genomic signatures of evolutionary transitions from solitary to group living. *Science*. 348:1139–1143.

Kocher SD, Paxton RJ (2014) Comparative methods offer powerful insights into social evolution in bees. *Apidologie*. 45:289–305.

Konovalov DA, Manning C, Henshaw MT (2004) kingroup: a program for pedigree relationship reconstruction and kin group assignments using genetic markers. *Molecular Ecology Notes*. 4:779–782.

Leadbeater E, Carruthers JM, Green JP, Rosser NS, Field J (2011) Nest inheritance is the missing source of direct fitness in a primitively eusocial insect. *Science*. 333:874–876.

Lin N, Michener CD (1972) Evolution of sociality in insects. *The Quarterly Review of Biology*. 47:131–159.

Linksvayer TA, Johnson BR (2019) Re-thinking the social ladder approach for elucidating the evolution and molecular basis of insect societies. *Current Opinion in Insect Science*. 34:123–129.

Michener CD (1985) From solitary to eusocial: need there be a series of intervening species? *Fortschritte der Zoologie* (Stuttgart). 31:293–305.

Meunier J, West SA, Chapuisat M (2008) Split sex ratios in the social Hymenoptera: a meta-analysis. *Behavioral Ecology* 19:382–390.

Nonacs P (2000) Measuring and using skew in the study of social behaviour and evolution. *The American Naturalist*. 156:577–589.

Nonacs P (2003) Measuring the reliability of skew indices: is there one best index? *Animal Behaviour*. 65:615–627.

Nonacs P (2017) Go high or go low? Adaptive evolution of high and low relatedness societies in social Hymenoptera. *Frontiers in Ecology and Evolution*. 5.

Nowak MA, Tarnita CE, Wilson EO (2010) The evolution of eusociality. *Nature*. 466:1057–1062.

Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. *Evolution*. 43:258–275.

R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (2020) https://www.R-project.org/.

Rehan SM, Leys R, Schwarz MP (2012) A mid-cretaceous origin of sociality in xylocopine bees with only two origins of true worker castes indicates severe barriers to eusociality. *PLOS ONE*. 7:e34690.

Rehan SM, Toth AL (2015) Climbing the social ladder: the molecular evolution of sociality. *Trends Ecology Evolution*. 30:426–433.

Richards MH, von Wettberg EJ, Rutgers AC (2003) A novel social polymorphism in a primitively eusocial bee. *Proceedings of the National Academy of Sciences*. 100:7175–7180.

Schwarz MP, Tierney SM, Rehan SM, Chenoweth LB, Cooper SJ (2011) The evolution of eusociality in allodapine bees: workers began by waiting. *Biology Letters*. 7:277–280.

Spessa A, Schwarz MP, Adams M (2000) Sociality in *Amphylaeus morosus*. *Annals of the Entomological Society of America*. 93:684–692.

Stark RE (1992) Cooperative nesting in the multivoltine large carpenter bee *Xylocopa sulcatipes* Maa (Apoidea: Anthophoridae): do helpers gain or lose to solitary females? *Ethology*. 91:301–310.

Szathmáry E, Smith JM (1995) The major evolutionary transitions. *Nature*. 374:227–232.

Ulrich Y, Perrin N, Chapuisat M (2009) Flexible social organization and high incidence of drifting in the sweat bee, *Halictus scabiosae*. *Molecular Ecology*. 18:1791–1800.

Wang J (2004) Sibship reconstruction from genetic data with typing errors. *Genetics*. 166:1963–1979.

West-Eberhard MJ (1996) Wasp societies as microcosms for the study of development and evolution. *Natural History and Evolution of Paper Wasps*, eds Turillazzi S & West-Eberhard MJ (Oxford University Press), pp 290-317.

Wilson EO (1971) The insect societies. The insect societies. Belknap Press of Harvard University Press.

Zheng X, Levine D, Shen J, Gogarten SM, Laurie C, Weir BS (2012) A high-performance computing toolset for relatedness and principal component analysis of SNP data. *Bioinformatics*. 28:3326–3328.

Table 1. Reproductive output and intracolony relatedness between social nestmates of *Amphylaeus morosus*. Reproductive skew values are calculated using the binomial skew index (*B*) and sex ratios are calculated as the proportion of male offspring in the colony. *If only one adult female was present at the time of collection, intracolony relatedness was estimated by back-calculating pedigrees to infer the relationship between adult nestmates based on the relationship of the remaining female to the offspring in the nest.

Nest	**Reproductive strategy**	**Reproductive output**		**Intracolony relatedness**	**Reproductive skew (*B*)**	**Sex ratio**
		Primary	**Secondary**			
5	Unrelated	3♀, 13♂	0	0*	0.469	0.813
13	Matrifilial	1♀, 12♂	0	0.486	0.462	0.923
35	Matrifilial	1♀, 9♂	0	0.511	0.444	0.889
36	Matrifilial	1♀, 11♂	0	0.512	0.458	0.917
55	Full-sibling	1♀, 5♂	0	0.699	0.417	0.857
59	Full-sibling	2♀, 4♂	0	0.750*	0.417	0.667
60	Full-sibling	2♀, 7♂	0	0.749	0.444	0.778
62	Full-sibling	3♀, 10♂	0	0.785	0.462	0.769
77	Full-sibling	3♀, 9♂	1♂	0.750*	0.319	0.769
145	Full-sibling	2♀, 11♂	0	0.653	0.462	0.846
Mean				**0.589**	**0.435**	**0.827**

Table 2. Comparison of colony productivity variables and inclusive fitness benefits between life-history strategies of *Amphylaeus morosus*. Parasitisation rate is the proportion of nests with at least one parasitised brood cell.

Reproductive strategy	**Sample size**	**Intracolony relatedness**		**Reproductive output**	**Parasitisation rate**	**Benefit of social nesting**	**Inclusive fitness**		**Genetic investment by secondary**
		Expected	**Observed**				**Primary**	**Secondary**	
Solitary	289	n/a	n/a	5.11±0.222	0.290±0.027	n/a	2.78±3.49	n/a	n/a
Matrifilial	3	0.5	0.503 (0.486 – 0.512)	12.33±1.202	0	8.89	11.67±0.882	4.45± 0.441	3.69±0.51
Full-sibling	6	0.75	0.722 (0.653 – 0.785)	10.33±1.406	0	7.39	10.17±1.327	5.54± 0.995	3.42±0.22

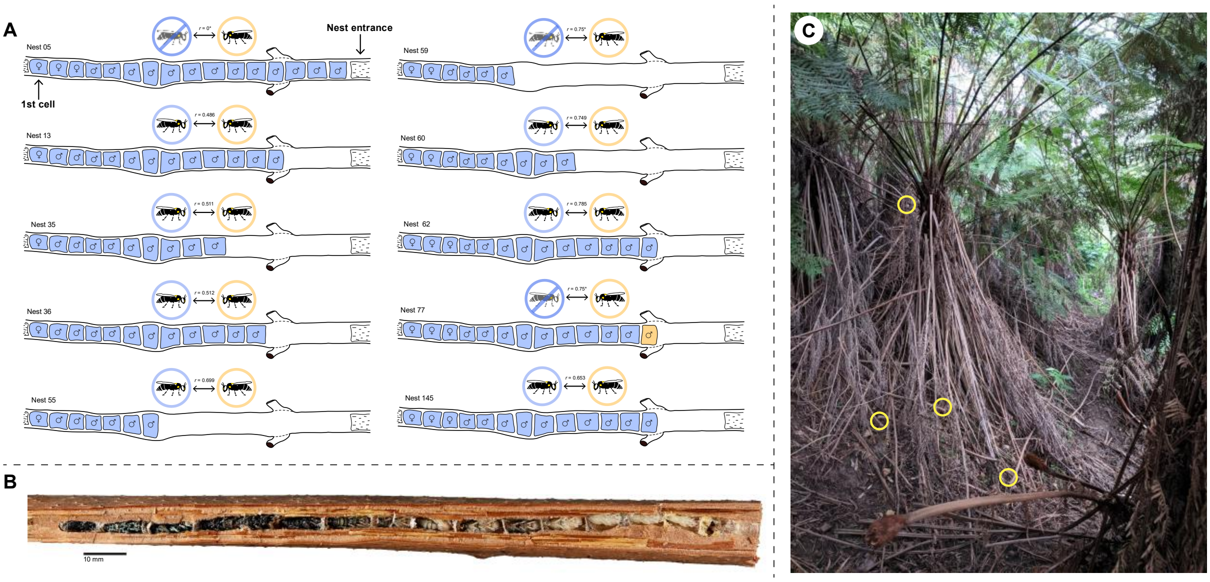

Figure 1. A. Visualisation of the reproductive skew for all sequenced social nests. Blue cells represent the primary females brood and orange represents the secondary females' brood. Adults that were not present in the nest at the time of collection but laid brood in the nest are denoted with a strike through the circle. B. Social nest with full clutch of offspring, showing sequential order of brood laying (image of nest 05). C. Nesting habitat of *Amphylaeus morosus* in the Dandenong Ranges, Victoria. Yellow circles show close proximity of nesting sites.

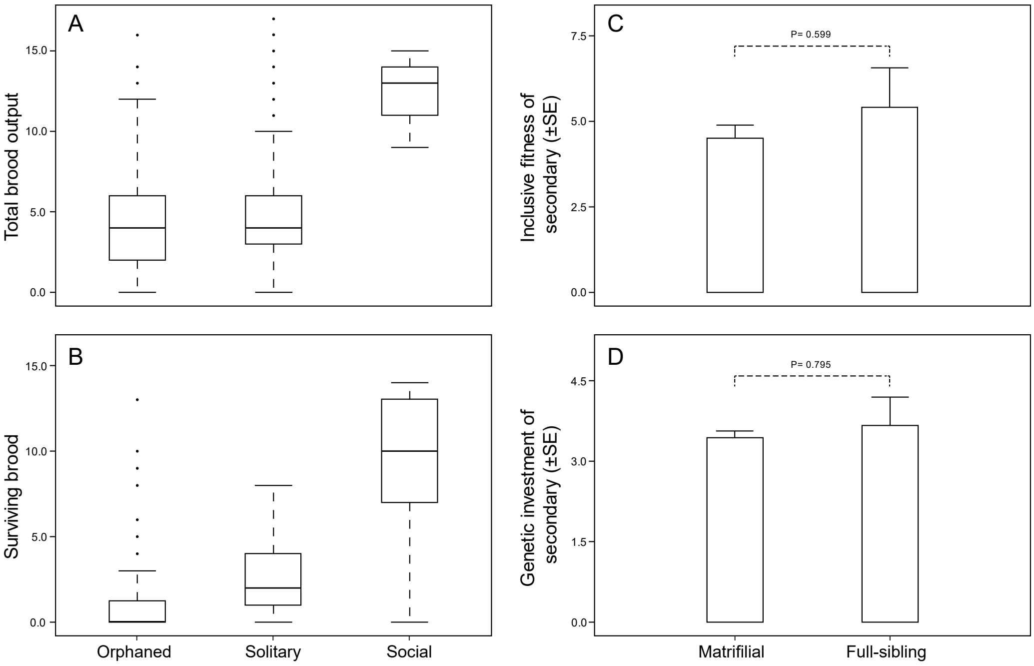

Figure 2. Comparison of A. total clutch size and B. total offspring that survived to adulthood between orphaned nests (n = 86), solitary nests (n = 203) and social nests (n = 13) of *Amphylaeus morosus*. C. Inclusive fitness of secondary females for each reproductive strategy. D. Mean genetic investment for secondary females in a nest for each reproductive strategy. Statistical significances using a Mann-Whitney test between matrifilial and full-sibling strategies are given.

5.11 Supplementary material

Appendix

Dataset S1 (separate file). Collection and SNP relatedness estimates for *Amphylaeus morosus* nests and individuals. Hearn et al. (2022).

Materials and methods

Nest collection

Nests of *Amphyaleus morosus* were collected from multiple locations within the Dandenong Ranges, Victoria. Nest collection localities in the Dandenong Ranges constituted semi-connected montane forests within a single, large population as evidenced by (Davies 2021). The frequency of social nesting did not differ depending on collection locality. The collection data is given in (Hearn et al. 2022).

DartR

A pilot analysis using four adult females was initially conducted with the Dartseq pipeline (Diversity Arrays Technology$^{©}$) to compare the SNP calls and read depth between head and thoracic tissue and to also determine the level of background contamination of non-genomic DNA. As there was no significant difference between the head and thoracic tissue, both tissue types were used. SNP loci were assigned the following DArT scores: “0” = reference allele homozygote, “1” = SNP allele homozygote, and “2” = heterozygote based on their allelic variation. Only one diploid male was detected, and this did not impact further analyses. Genotype frequencies did not significantly differ from those expected under Hardy-Weinberg equilibrium. The inbreeding coefficient was estimated as $F_{\text{is}} = 0.007$ (Table S1).

COLONY

The input parameters set in the program COLONY (Jones and Wang 2010) were as follows: Mating system – female polygamy, male polygamy; no inbreeding; species – dioecious and haplodiploid; Length of run – long; Method – Full-Likelihood (FL); Likelihood Precision – High. Marker type was set to codominant for all loci with a genotyping error rate of 0.001, which was calculated in the package SNPRelate in R. Adult females in the nest at the time of collection were assumed to be mothers of the brood and were therefore given a 0.9 probability that at least one female in the candidate list was a mother.

Pairwise relatedness

We used the updated allele frequencies generated by COLONY to estimate within nest relatedness in KINGROUP V2 (Konovalov et al. 2004). Pairwise relatedness among adult female nestmates was estimated for 10 nestmate pairs. In cases where nestmates had left the nest prior to collection, pairwise relatedness between adult nestmates was inferred from the relationship between the remaining nestmate and the offspring in the nest (inferred colonies $N = 3$, measured colonies $N = 7$). Pairwise relatedness was also estimated among offspring and between adult nestmates and offspring. However, due to some conflicting results with the ability of KINGROUP V2 to accurately calculate asymmetrical relatedness we conducted further analyses to confidently assess relatedness in this haplodiploid species.

We confirmed the pairwise relatedness between adult nestmates and between nestmates and offspring in five separate programs (KINGROUP, KINSHIP, RELATEDNESS, COANCESTRY, RELATED) using the Queller and Goodnight (1989) estimator. We used this estimator as it is the only estimator available across numerous independent software that truly accounts for the asymmetrical relatedness present in haplodiploid pedigrees. We used the programs KINSHIP and RELATEDNESS (Goodnight and Queller 1999) to calculate pairwise relatedness values across both haploid (male) and diploid (female) genotypes. We used the program COANCESTRY (Wang 2011) to assess the pairwise relatedness between adult females with diploid genotypes and, the R package, RELATED (Pew et al. 2015) was used to calculate 95% confidence intervals for each pairwise estimate to determine if the upper and lower limits could explain the unexpected estimates. Expected distributions of pairwise relatedness values were generated for specific pedigree relationships based on observed allele frequencies (Fig S2). These expected values were based on 2,000 simulated values using allele empirical frequencies based on the assayed *A. morosus* adult females, with zero representing the average relatedness between any two individuals randomly drawn from within the sampled population.

Measuring reproductive skew

Reproductive skew was calculated using SKEW CALCULATOR 2003 © Peter Nonacs. We used the binomial skew index (*B*) (Nonacs 2000) which tests within each group if the observed variance in reproduction significantly differs from the expected variance if all group members have an equal probability to reproduce. This value can range from -1 indicating an equal share of reproduction to 1 which indicates complete monopoly over reproduction. For this index, a value of 0 indicates a random distribution of reproduction among group members. As the *B* index accounts for the time an individual spends in the group, we made the assumption that all members were equally present in the group given that, in most cases, nestmates were kin utilising their natal nest and were also still present at the time of collection when brood laying had finished. Significance ($\alpha = 0.05$) and confidence (CI = 95%) levels for each *B* value were calculated from 10,000 simulations.

We assessed the estimates generated for the eight most commonly used skew indices (S index (*S*) (Reeve and Ratnieks 1993); Corrected S index (S_c) (Keller and Krieger 1996); Effective number S index (S_3) (Pamilo and Crozier 1996); Monopolisation index (*Q*) (Ruzzante et al. 1996); Iterative skew index (λ) (Kokko and Lindstrom 1997); Morisita index (I_δ) (Tsuji and Tsuji 1998); Standardised Morisita index (I_p) (Tsuji and Tsuji 1998) and; Binomial index (*B*) (Nonacs 2000)) and deemed the *B* index the most appropriate (Table S3). Settings: 1,000 simulations, 95% CI, α=0.05, equal length of time in nest assumed.

Inclusive fitness calculations

To determine indirect fitness estimates for social secondaries, we calculated the benefit of social nesting as the difference between the mean number of offspring that survived to adulthood in social nests for matrifilial and full-sibling strategies and the mean number of offspring that survived to adulthood in solitary nests (mean±SEM = 2.78±3.49). The benefit of social nesting was then multiplied by the pairwise relatedness between the social secondary and social primary, which, for simplicity, was taken as the expected relatedness for each strategy (i.e. mother-daughter = 0.5; full-sisters = 0.75). When actual calculated relatedness values were used to calculate inclusive fitness, the results did not change. Direct fitness estimates for social primaries were taken as the number of offspring they laid.

To determine if the relative reproductive value of each sex in social nests significantly altered the indirect benefits gained by social secondaries, we separately calculated the genetic investment based on the opposing assumptions that 1) both male and female offspring are equally as valuable and 2) that female offspring are twice as valuable as male offspring.

Genetic investment of the secondary female was calculated as the total number of the primary female's offspring in the nest of each sex (N_{sex}) multiplied by their degree of relatedness to the secondary female (r_s) (i.e. $N_{female} \times r_s + N_{male} \times r_s$). The relative reproductive values (ν) were then incorporated as follows for both matrifilial and full-sibling strategies (Table S4):

Matrifilial

$$\frac{\mathrm{N_{female}}}{\nu_{\mathrm{female}}} \times r_s + \frac{\mathrm{N_{male}}}{\nu_{\mathrm{male}}} \times r_s$$

Full-sibling

$$\left(\frac{\mathrm{N}_{\mathrm{female}}}{\nu_{\mathrm{female}}} + \frac{\mathrm{N}_{\mathrm{male}}}{\nu_{\mathrm{male}}}\right) \times r_{\mathrm{s}}$$

When incorporating the relative reproductive value of each sex into these calculations no statistical difference between reproductive strategies was detected (Mann-Whitney: U = 5.00, *P* = 0.302; Table S4).

Sex ratios

Amphylaeus morosus females were 1.11 times heavier than males (female brood: 61.31 ± 0.65 mg, *n* = 392; male brood: 55.05 ± 0.69 mg, *n* = 516; two-way ANOVA: $F_{1,\,856}$ = 40.791, *P* <0.001; Table S5). Development times for females and males from egg to adult can range from 30-50 days (Hearn pers. obs).

Hierarchal dissections

Materials and Methods

Hierarchal analyses

We used Monte Carlo resampling techniques to determine if potential reproductive dominance hierarchies were based on morphological characteristics (See da Silva et al. 2015; Dew et al. 2018; Spessa et al. 2000). To do this, we simulated social nest pairings to investigate whether social pairs showed differences in body size, wing wear and ovary size that were greater than those expected for solitary nesting females. In social nests, females were ranked based on their body size, wing wear and ovary size (greatest to smallest), these social nests were pooled together and simulated random ‘nestmate’ pairs drawn together and compared to calculate expected nestmate differences. This procedure was repeated 10000 times for each variable to generate null distributions and determine the proportion of these distributions that exceeded the observed mean difference. Unless otherwise stated, values are presented as mean±SE.

Results

Dominance hierarchies

To account for the low sample size, we used Monte Carlo simulations to further explore any morphological differences between ovary size, body size and wing wear that might infer the presence of dominance hierarchies (Table S6). From the 10000 simulated social pairs, 7722 (77.22%) showed differences in ovary size greater than the observed mean difference of 0.311 ± 0.059 mm between two female nestmates and when repeated with individuals from the solitary pool, 7472 (74.72%) had greater differences than the observed mean difference. These results were similar for body size, with 8922 (89.22%) simulated pairs drawn from the social pool having a difference greater than the observed mean difference of 0.468 ± 0.13 mm between two female nestmates and 3144 (31.44%) simulated pairs drawn from the solitary pool having a greater difference. Only 1556 (15.56%) simulated social pairs showed differences in wing wear greater than the observed mean difference of 18.33 ± 5.37 (wing nicks) and only 525 (5.25%) simulated pairs drawn from the solitary pool.

We used a linear regression with a Gaussian distribution to determine if any morphometric variables (body size, wing wear and ovary size) were correlated with reproductive ability, which was measured as the number of brood cells laid. Reproductive ability was not correlated with ovary size or body size ($F = 0.042$, d.f. = 1, 19, $P = 0.840$; $F = 0.033$, d.f. = 1, 19, $P = 0.857$) respectively, but was significantly correlated with wing wear ($F = 4.886$, d.f. = 1, 19, $P = 0.039$).

Division of labour

Our hierarchal analyses of multifemale nests in *A. morosus* suggest an absence of morphological castes. We found no evidence of morphological hierarchies based on ovary size and body size in *A. morosus*. These findings corroborate those of Spessa et al. (2000), who found that ovary size and body size did not predict unequal sharing among social nestmates in *A. morosus*. However, in multifemale nests there was a clear dominance rank between dyadic females based on foraging activity and reproduction. In all cases, the reproductive primary female appeared to also be the primary forager and likely provisioned her own cells, suggesting that the secondary female remains in the nest. Evidence for matrifilial colonies was further supported by the discrepancy in wing wear between mother and daughters. In these nests, mothers had extremely worn wings indicating extended age when compared to other social dominants with similar reproductivity.

Table S1. Mean observed heterozygosity (Ho), mean expected heterozygosity (He) and inbreeding coefficient for 947 loci across 31 sampled adult female *Amphylaeus morosus.*

No. individuals	No. loci	Observed heterozygosity (Ho)	Expected heterozygosity (He)	Inbreeding coefficient
31	947	0.159	0.159	0.0074

Table S2. Comparisons of expected and observed intracolony relatedness values for solitary and social nests of *Amphylaeus morosus*. Observed mean relatedness for each pairwise relationship was estimated from 950 polymorphic loci with 95% confidence intervals calculated from 1000 bootstrap samples. **N* = number of nests; *n* = number of individuals.

	Relationship	**Expected relatedness**	**Observed relatedness (95% CI's)**	***N***	***n***
Solitary mother and offspring	**Mother-daughter**	0.5	0.489 (0.464 – 0.511)	20	26
	Full-sisters daughters	0.75	0.648 (0.594 – 0.695)	20	49
Social nestmates	**Matrifilial**	0.5	0.503 (0.486 – 0.512)	6	6
	Full-sibling	0.75	0.722 (0.653 – 0.785)	3	3

Table S3. Reproductive skew values for the eight most commonly used skew indices. S index (S) (Reeve and Ratnieks 1993); Corrected S index (S_c) (Keller and Krieger 1996); Effective number S index (S_3) (Pamilo and Crozier 1996); Monopolisation index (Q) (Ruzzante et al. 1996); Iterative skew index (λ) (Kokko and Lindstrom 1997); Morisita index (I_δ) (Tsuji and Tsuji 1998); Standardised Morisita index (I_p) (Tsuji and Tsuji 1998) and; Binomial index (B) (Nonacs 2000). *P*-values are given for the binomial skew index. Bold *P*-values indicate a significant positive reproductive skew relative to the random distribution of reproduction in a group.

		Reproductive skew indices							**Binomial index**			
Nest	**Adult females**	S	S_c	S_3	Q	λ	I_δ	I_p	B	***P*-value**	**CI**	
5	2	1	1	1	1	1	2	1	0.469	**<0.001**	0.325	0.469
13	2	1	1	1	1	1	2	1	0.462	**<0.001**	0.294	0.462
35	2	1	1	1	1	1	2	1	0.444	**0.004**	0.228	0.228
36	2	1	1	1	1	1	2	1	0.458	**0.001**	0.280	0.280
55	2	1	1	1	1	1	2	1	0.417	**0.027**	0.144	0.144
59	2	1	1	1	1	1	2	1	0.417	**0.029**	0.143	0.143
60	2	1	1	1	1	1	2	1	0.444	**0.002**	0.227	0.227
62	2	1	1	1	1	1	2	1	0.462	**<0.001**	0.294	0.294
77	2	0.716	0.69	0.835	0.692	0.917	1.692	0.769	0.319	**0.004**	0.173	0.425
145	2	1	1	1	1	1	2	1	0.462	**<0.001**	0.289	0.289
Mean	**2**	**0.972**	**0.969**	**0.983**	**0.969**	**0.992**	**1.97**	**0.977**	**0.435**	<0.001		

Table S4. Indirect fitness, genetic investment of secondary female and reproductive values for nests of each reproductive strategy. Reproductive values (ν) for male and female offspring when the relative value of sexes is equal ($\nu_m = \nu_f$) and when males are half the value of females ($\nu_m = ½(\nu_f)$).

Nest	**Reproductive strategy**	**Expected Intracolony relatedness**	**Indirect fitness of secondaries**	**Average relatedness of secondary to primaries offspring**	**Genetic investment for secondary**	**Reproductive value (ν)**	
						$\nu_m = \nu_f$	$\nu_m = ½(\nu_f)$
5	Unrelated	0	0	0	0	0	0
13	Matrifilial	0.5	4.445	0.295	3.75	3.75	6.75
35	Matrifilial	0.5	4.445	0.313	3	3	5.25
36	Matrifilial	0.5	4.445	0.3	3.5	3.5	6.25
55	Full-sibling	0.75	5.543	0.375	2.25	2.25	4.125
59	Full-sibling	0.75	5.543	0.375	2.25	2.25	3.75
60	Full-sibling	0.75	5.543	0.375	3.375	3.375	6
62	Full-sibling	0.75	5.543	0.375	4.875	4.875	8.625
77	Full-sibling	0.75	5.543	0.375	4.5	4.5	7.875
145	Full-sibling	0.75	5.543	0.375	4.875	4.875	9

Table S5. Comparison of productivity and morphological variables between adult females in social and solitary colonies of *Amphylaeus morosus*. Sex ratios are presented as the mean numerical ratio for each colony type.

Colony type	Mean reproductive output	Offspring sex ratio	Mated status	Mean ovary size (mm)	Mean wing length (mm)	Mean wing wear
Solitary	5.14 ± 0.219	0.348 ± 0.039	Yes	1.132	5.702	7.00
Social	12.2 ± 0.725	0.834 ± 0.024	Yes	0.954	5.743	9.65

Table S6. Dissection data for all social genotyped nests. Ovary size is taken as the average length of the three largest oocytes, excessive wing wear was scored as 40+ as it was impossible to distinguish individual nicks and tears.

Nest	Adult female	Reproductive output	Mated status	Ovary size (mm)	Wing length (mm)	Wing wear	Hierarchal status
5*	1	0	Yes	0.839	6.179	0	Secondary
13	1	0	Yes	0.699	5.830	0	Secondary
13	2	13	Yes	1.196	6.063	40+	Primary
35	1	0	Yes	1.305	6.179	1	Secondary
35	2	9	Yes	1.833	6.296	40+	Primary
36	1	0	Yes	1.025	6.063	0	Secondary
36	2	12	Yes	1.507	5.713	40+	Primary
59*	1	0	Yes	0.979	6.296	6	Secondary
60	1	0	Yes	1.243	6.296	1	Secondary
60	2	9	Yes	0.854	5.713	9	Primary
62	1	0	Yes	0.901	6.413	5	Secondary
62	2	13	Yes	1.196	5.713	17	Primary
145	1	0	Yes	0.747	5.364	0	Secondary
145	2	13	Yes	1.009	6.779	6	Primary

*Social genotyped nest with only one adult female present at the time of collection. Hierarchal status of these adult females is inferred from the observed patterns of the other known nests.

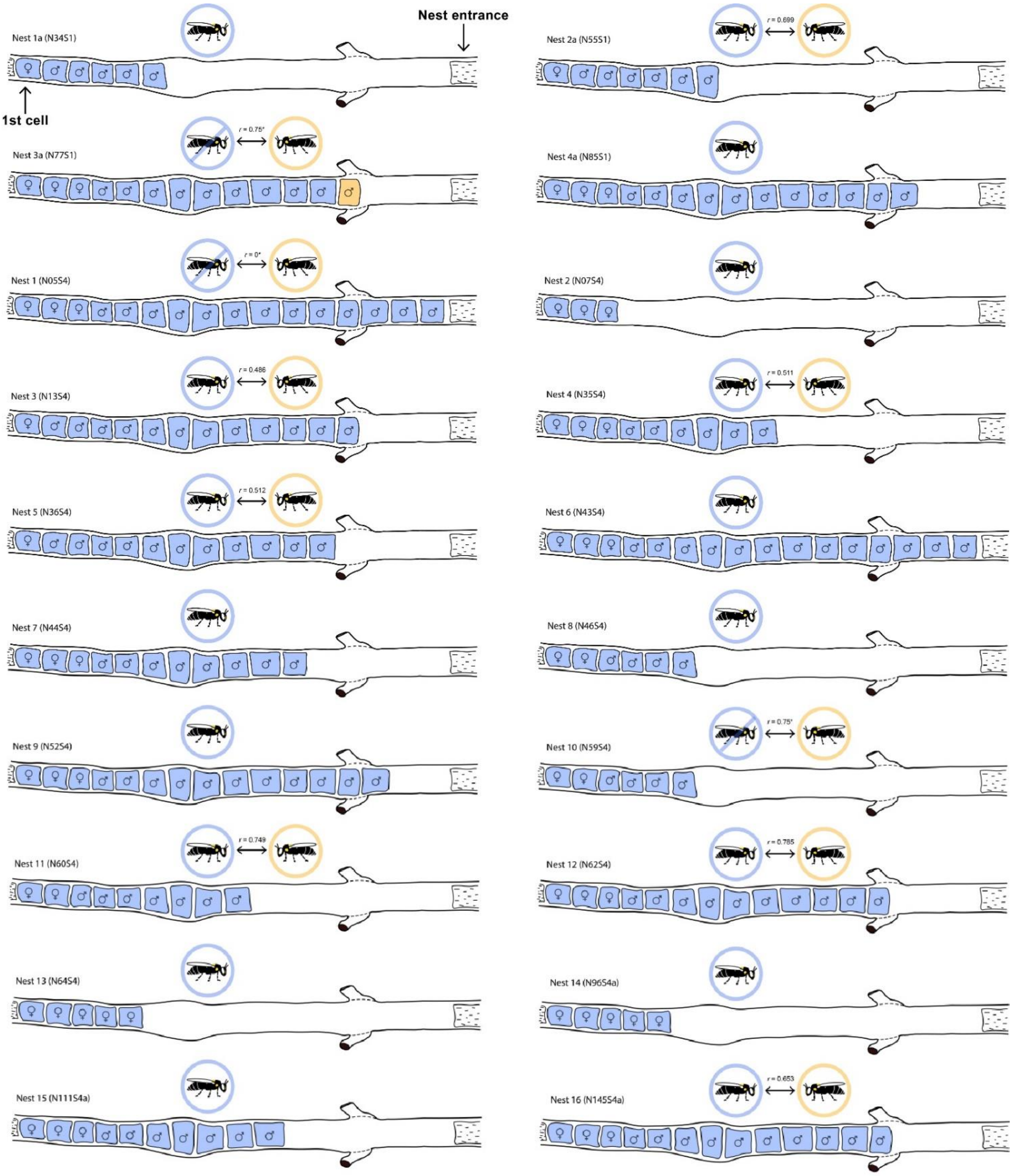

Figure S1. Visualisation of the reproduction contribution for all sequenced nests. Adults that were not present in the nest at the time of collection but laid brood in the nest are denoted with a strike through the circle.

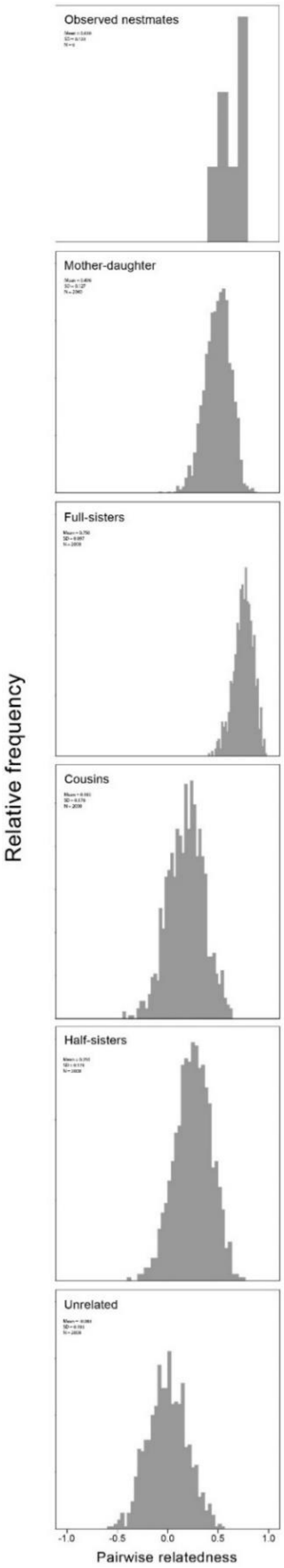

Figure S2. Frequency histograms of observed intracolony relatedness of *Amphylaeus morosus* females in social nests and expected pairwise relatedness estimates for specific pedigree relationships of adult *A. morosus* females. Pedigree distributions are based on 2000 simulated values calculated from the same allele frequencies (950 loci) as the observed estimates.

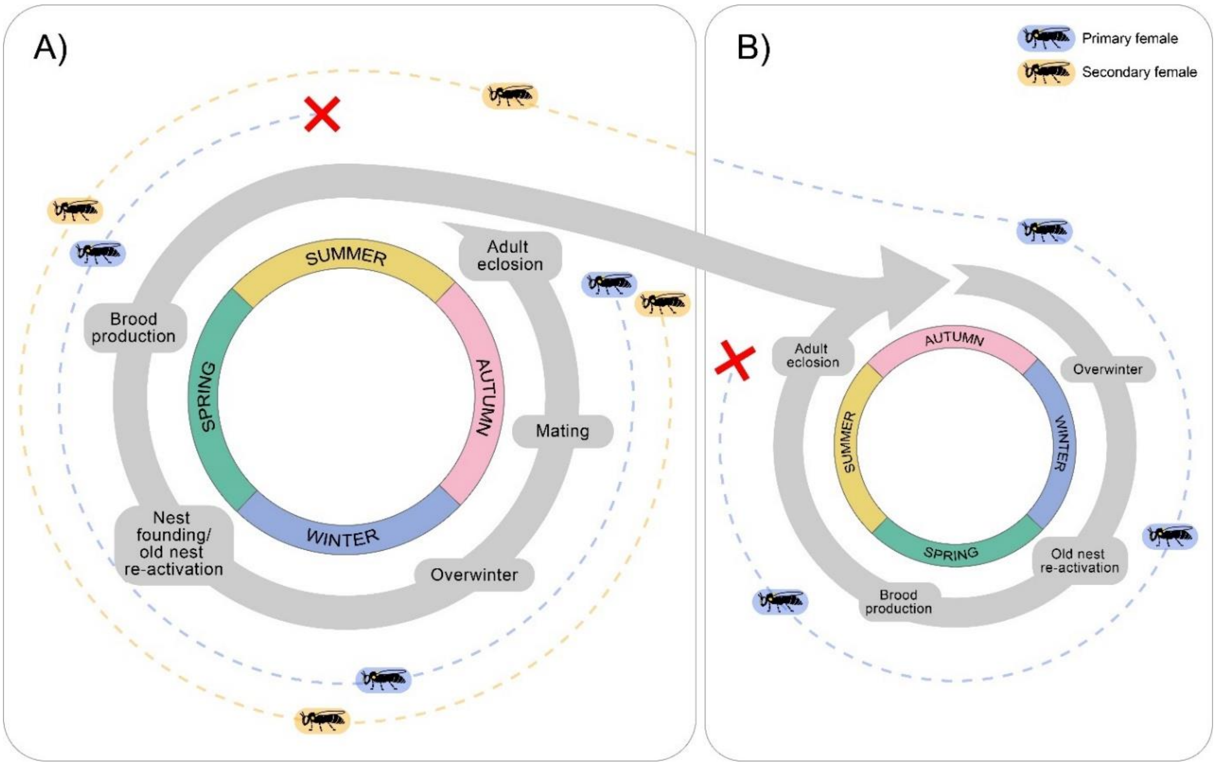

Figure S3. Semelparous life-cycle for *Amphylaeus morosus* primary and secondary females. A) Univoltine life-cycle for females that only live for one season and first year for non-reproductive secondary females. B) Life-cycle of females that persist into a second season by re-using a natal nest to become reproductive primaries.

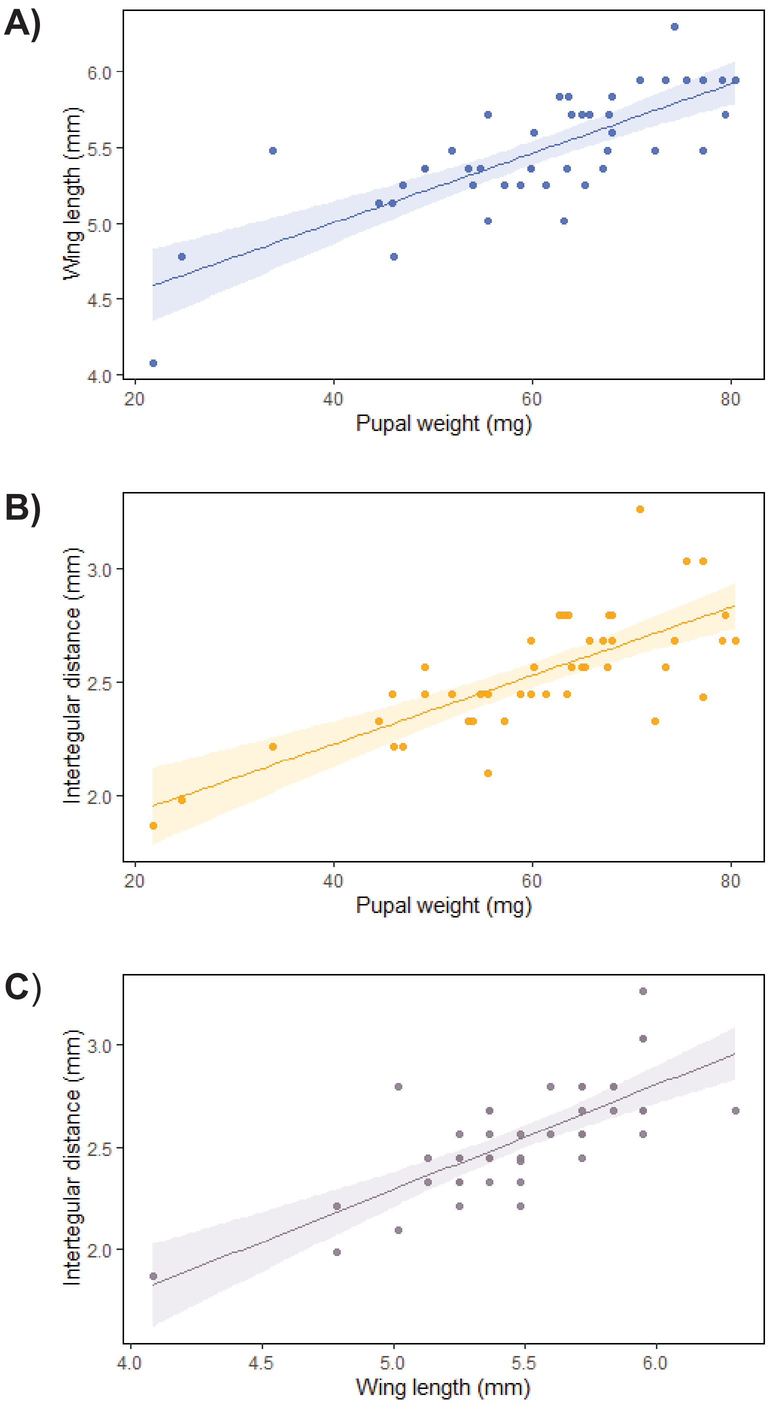

Figure S4. Relationships between three body size variables. Measurements were taken from the mature brood of sequenced nests (*N* = 44) and showed moderate-strong R squared coefficients; A) $R^2 = 0.601$; B) $R^2 = 0.607$; C) $R^2 = 0.567$.

References

da Silva CRB, Stevens MI, Schwarz MP (2015) Casteless sociality in an allodapine bee and evolutionary losses of social hierarchies. *Insectes Sociaux*. 63:67–78.

Davies OK (2021) Mitochondrial heteroplasmy in Australian native bee *Amphylaeus morosus* and its association with the parasite *Wolbachia*, Ph.D. thesis, Flinders University, Adelaide.

Dew R, Tierney S, Gardner M, Schwarz M (2018) Casteless behaviour in social groups of the bee *Exoneurella eremophila*. *Apidologie*. 49:265–275.

Goodnight KF, Queller DC (1999) Computer software for performing likelihood tests of pedigree relationship using genetic markers. *Molecular Ecology*. 8:1231–1234.

Hearn LR, Davies OK, Schwarz MP (2022) Extreme reproductive skew at the dawn of sociality is consistent with inclusive fitness theory but problematic for routes to eusociality. Dryad, Dataset. https://doi.org/10.5061/dryad.wdbrv15r5.

Jones OR, Wang J (2010) COLONY: a program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*. 10:551–555.

Keller L, Krieger MJ (1996) Mating success of male birds. *Nature*. 380:208–209.

Kokko H, Lindstrom J (1997) Measuring the mating skew. *The American Naturalist*. 149:794–799.

Konovalov DA, Manning C, Henshaw MT (2004) kingroup: a program for pedigree relationship reconstruction and kin group assignments using genetic markers. *Molecular Ecology Notes*. 4:779–782.

Nonacs P (2000) Measuring and using skew in the study of social behaviour and evolution. *The American Naturalist*. 156:577–589.

Pamilo P, Crozier RH (1996) Reproductive skew simplified. *Oikos*. 75:533–535.

Pew J, Muir PH, Wang J, Frasier TR (2015) related: an R package for analysing pairwise relatedness from codominant molecular markers. *Molecular Ecology Resources*. 15:557–561.

Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. *Evolution*. 43:258–275.

Reeve HK, Ratnieks F (1993) Queen-queen conflicts in polygynous societies: mutual tolerance and reproductive skew. Oxford University Press.

Ruzzante DE, Hamilton DC, Kramer DL, Grant JW (1996) Scaling of the variance and the quantification of resource monopolization. *Behavioral Ecology*. 7:199–207.

Spessa A, Schwarz MP, Adams M (2000) Sociality in *Amphylaeus morosus*. *Annals of the Entomological Society of America*. 93:684–692.

Tsuji K, Tsuji N (1998) Indices of reproductive skew depend on average reproductive success. *Evolutionary Ecology*. 12:141–152.

Wang J (2011) Coancestry: a program for simulating, estimating and analysing relatedness and inbreeding coefficients. *Molecular Ecology Resources*. 11:141–145.

Chapter 6 – The decision to guard vicariously drives split sex ratios in a facultatively social bee

Lucas R. Hearn[1*], Mark I Stevens[2,3] and Michael P. Schwarz[1]

[1]*College of Science and Engineering, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia.*

[2]*Earth and Biological Sciences, South Australian Museum, GPO Box 234, Adelaide, SA 5001, Australia.*

[3]*School of Biological Sciences, University of Adelaide, SA 5005, Australia.*

*Corresponding author: Lucashearn7@gmail.com

6.1 Abstract

It has been argued that in social Hymenoptera, split sex ratios may facilitate altruistic behaviour through female-biased sex ratios that increase the indirect fitness gained by workers. However, this view remains contentious, despite its prominence in early studies of eusociality in haplodiploids. Almost no attention has been directed to situations where split sex ratios may potentially decrease the payoffs for worker-like behaviour, increasing selective thresholds for eusociality. We examined sex ratios in a facultatively social bee, *Amphylaeus morosus*, which sequentially provisions cells in a linear tunnel. Mothers in this species adjust brood sex ratios depending on the presence of a nest guard and in a pattern that contradicts the traditional thinking of split sex ratios. Whilst the production of daughters was constant across social and solitary nests, socially nesting mothers produced more brood when a non-reproductive guard was present, but these extra brood were all male. This leads to split sex ratios, vicariously driven by guards that are unable to manipulate sex ratios in their favour. Importantly, if guarding becomes more common this would lead to an excess of males and lower the genetic value of these extra males to guards, effectively putting a brake on selection for worker-like behaviour to spread throughout the population.

Key words: Sex allocation, Split sex ratios, Altruism, Eusociality, Guarding, Hymenoptera

6.2 Introduction

Fisherian principles state that under most conditions, natural selection should favour equal investment into sons and daughters leading to balanced population investment ratios (Fisher 1930). At the same time, individual parents should bias their offspring towards the sex that will generate the greatest return on their individual investments (Trivers and Willard 1973). In haplodiploid populations, males develop from an unfertilised egg, such that mated females can control the sex of their offspring during oviposition; this flexibility allows mothers to skew sex allocation ratios in response to ecological, social or physiological cues (Trivers and Hare 1976). In some cases, sex biased ratios can arise under conditions that drive individual deviations from a Fisherian parity (Grafen 1986). In extreme scenarios, split sex ratios can occur due to factors such as local mate competition (Hamilton 1967), unmatedness (where some mothers can only produce male brood, Godfray 1990), and situations where sons' or daughters' fitness's vary with maternal condition (e.g. Clutton-Brock et al. 1982). However, the most intriguing cases occur in haplodiploids, such as ants, bees and wasps, where mothers can readily control the sex of their offspring and where relatedness between sisters and brothers is asymmetrical and indirect payoffs for worker-like behaviour will vary with sex ratio (Gardner et al. 2012).

Inclusive fitness theory predicts that workers in eusocial hymenopteran societies should favour rearing sisters over brothers because haplodiploidy results in a much higher relatedness to the former ($r = ¾$) than the latter ($r = ¼$) (Hamilton 1964*a*,*b*). In contrast, queens should generally prefer to invest equally in daughters and sons because of Fisherian dynamics (Fisher 1930; MacArthur 1965), and this difference in preferred sex allocation strategies can lead to queen-worker conflict (Alpedrinha et al. 2014; Trivers and Hare 1976). Queen-worker conflict can manifest in situations where workers are able to preferentially rear sisters, for example by killing brothers (Kümmerli and Keller 2009). Both workers and queens in these societies have means to manipulate sex ratios in their favour to enhance their inclusive fitness; workers by directing resources towards female progeny and queens by laying less female progeny, resulting in split sex ratios (Mueller 1991; Rosset and Chapuisat 2006). This power struggle suggests that both worker and queen may have incomplete control over sex allocation at both the population and colony level, but ultimately an equal (Fisherian) investment or female-biased investment is favoured (Hamilton 1972; Meunier et al. 2008).

Sex-biased ratios have been shown to sometimes lower the benefits needed for worker altruism to persist under haplodiploidy, but whether this mechanism is strong enough to facilitate the evolution of eusociality is contested (da Silva 2022; Gardner et al. 2012; Rautiala et al. 2019). While many studies have attempted to demonstrate how female-biased sex ratios can lower thresholds for sib-rearing (Boomsma and Grafen 1991; Frank and Crespi 1989), few have explored whether split sex ratios can act to raise the thresholds for worker altruism to become a widespread strategy.

Sex ratios in social colonies are often skewed by worker help, either directly, by manipulating the sex of the queens' eggs or, indirectly, by enhancing the provisioning capacity of queens (Frank and Crespi 1989). In the latter case, social mothers should invest into the sex with the higher reproductive value. This situation becomes amplified in mixed populations of solitary and social nesters. If solitary mothers lay more philopatric offspring (females) as posited by the “Constant Philopater” hypothesis (Rodrigues and Gardner 2016), then social mothers should capitalise by producing more male offspring (da Silva 2022). Investigating these mechanisms may offer a unique perspective on the role of sex ratio variation in the transition from lone mothers to females that help rear siblings (Trivers and Hare 1976).

Amphylaeus morosus (Smith, 1879) (Hymenoptera: Colletidae) is a stem-nesting, mass-provisioning bee species with large facultatively social populations throughout the montane regions of south-eastern Australia. Mothers produce one brood per year in which nests are provisioned linearly in an unbranched tunnel (Spessa et al. 2000). Social colonies of this species may contain up to three adult females but generally colonies consist of two adult females that may form either matrifilial or full-sibling pairings (Hearn et al. 2022). In these familial colonies, reproduction is monopolised by one female that forages and provisions her own brood while the non-reproductive, guard female, passively defends the nest from potential invaders. Despite this monopoly over reproduction in social nests, guards are always mated and able to disperse and reproduce on their own as solitary foundresses (Hearn et al. 2022).

We use this stem-nesting species, involving defined guard and reproductive roles, to investigate three key questions that challenge long-standing views relating to sex allocation and kin selection theories in haplodiploid insect societies. Specifically, we ask: 1) Why are a set number of females provisioned first in solitary and social nests? 2) does the presence of a nest guard influence sex ratios in a facultatively social population? 3) What determines population-wide investment sex ratios when individual strategies deviate from parity? The answers to these questions could provide insights into why sociality in *A. morosus* is maintained at such a low frequency and show that split sex ratios can raise the selective thresholds for eusociality.

6.3 Methods

Nest collection

Intact nests of *Amphyaleus morosus* (n = 298) were collected from the Dandenong Ranges, Victoria, Australia throughout the reproductive season, which ranges from late-spring (November) to late summer (February). Nests were sampled across five consecutive years (2017-2021; four reproductive seasons) and seven separate collections. Within the Dandenong Ranges, *A. morosus* nests in abscissed fronds of the rough fern tree, *Cyathea australis* R.Br. Domin. Nests were collected during the morning in episodes of light rain or cool temperatures to ensure there was no extra-nidal activity and colonies were fully intact. Nest entrances were sealed and all nests stored on ice in insulated boxes and transported to the laboratory where they were stored at 10°C until processed.

Colony data

In total, 193 nests had at least one viable host brood that survived to adulthood, while 103 nests were extirpated by parasites or failed due to other unknown causes. Nests were opened longitudinally up to the first brood cell so that mothers and any additional contents preceding the first brood cell could be removed and placed in 99% ethanol. Where possible, immature brood were left in their cells and reared to pupation at ambient room temperature. The reared nests were opened once the first cell had matured to medium stage pupae (pupal colour with some pigmentation), indicating nests had reached a point where all brood were mature enough to visibly sex but not so mature that they had eclosed to adults and broken through their cell partitions.

Sex allocation

To assess patterns of investment sex ratios in colonies of *A. morosus*, brood sex, wet weight, and brood cell position were recorded. Pupae were weighed on a Thermoline precision balance to ± 0.1 mg. The numerical sex ratio (NSR) was calculated as the number of male brood divided by the total number of brood that reached pupation to a point that they could be reliably sexed ($\text{NSR} = \Sigma_{\text{male brood}} / \Sigma_{\text{male brood + female brood}}$). Investment sex ratio (ISR) was calculated as a product of the numerical sex ratio and the pupal weight ratio calculated from mean brood sex pupal weight and was used to test whether the observed numerical sex ratio deviated from the investment a mother allocates to each sex. Brood that had died before nests were opened were not included in pupal weight measurements but were used for numerical sex ratio calculations. To examine sex allocation patterns across cell positions in the nest, cell position was coded so that ‘cell 1’ corresponded to the first cell provisioned (furthest from the nest entrance), following Hearn et al. (2022).

Statistical analyses

Premature mortality is common in adult females of this species during brood provisioning, causing potentially confounding factors when categorizing colony size. To account for this as best as possible, all orphaned nests (zero adult females at the time of collection) and single-female nests were classified as solitary and all nests with at least two adult females classified as social. For some analyses, brood that reached adulthood were pooled across all nests and the pupal sex was treated as a binomial response variable (female = 0, male = 1).

All statistical analyses were performed in SPSS version 27.0 (IBM, Armonk, NY) and R version 4.0.4 (R Core Team, 2018). Tests of normality and homoscedasticity were assessed using a Shapiro–Wilk test and Levene's test. A Chi-square goodness of fit test was used to determine if our observed numerical sex ratio significantly differed from an expected null hypothesis ratio calculated from the investment sex ratio. Where necessary we used arcsine back-transformed values of sex ratio and the corresponding confidence intervals. Unless otherwise stated, values are presented as mean ± SE.

6.4 Results

Population-wide sex allocation

We collected 193 viable *Amphylaeus morosus* nests containing 392 female brood and 516 male brood in total. On average, females were 1.11 times heavier than males (female brood: 61.31 ± 0.65 mg; male brood: 55.05 ± 0.69 mg; independent samples t-test: $F_{858} = 15.926$, $P < 0.001$). The investment sex ratio was slightly male biased and significantly differed from an expected 1:1.11 female:male ratio (ISR = 0.523, $n = 908$). Mean pupal weight for each sex did not differ between solitary and social colonies (female brood: independent samples t-test: $F_{363} = 3.640$, $P = 0.057$; male brood: independent samples t-test: $F_{493} = 0.008$, $P = 0.929$; Table 1).

To account for any nest effects that may influence sex ratios across sampling periods, we analysed the proportion of solitary and social nests as a function of sampling period. The number of social nests collected per sample ranged from zero to eight compared to solitary nests, which ranged from six to 78 (Table S8), but the proportion of each colony type collected across samples did not differ (Fishers exact test: $P = 0.312$). There was also no difference in the mean numerical sex ratio for solitary and social nests across sampling periods (solitary: Kruskal-Wallis: $H = 5.579$, d.f. = 5, $P = 0.349$; social: Kruskal-Wallis: $H = 1.109$, d.f. = 2, $P = 0.574$; *SI Appendix*).

Sex ratio across cell position

The population numerical ratio showed a positively increasing sigmoidal trend as cell position increased (Fig 1). In highly productive nests (>7 brood cells), female brood occurred primarily in the first set of cells (cell positions 1-7; NSR = 0.249, χ^2= 100.50, *P* <0.001), whereas numerical ratio of the later cells was entirely male-biased (cell positions 8-17; NSR = 1.0; Table 2). There was no difference in brood sex placement across cell position between social and solitary nests (Fig 1).

Split sex ratios

Variation in numerical ratios between nests with different colony sizes and nests with different architecture was noticeable. Nests of *A. morosus* showed split sex ratios between both solitary and social colonies (Table 1). The numerical sex ratio was significantly correlated with social status (r_s = 0.305, *P* <0.001). Social nests were heavily male biased (social NSR = 0.850), where the sex ratio across solitary nests was female-biased (solitary NSR = 0.338; Table 1). The number of female offspring produced across both solitary and social nests remained constant (mean female offspring: solitary = 1.96 ± 0.09, social = 1.86 ± 0.29; Mann-Whitney test: U = 1235.0, *P* = 0.872), but male offspring production increased significantly when more than one adult female was present in the nest (mean male offspring: solitary = 2.16 ± 0.25, social = 9.00 ± 0.71; Mann-Whitney test: U = 207.5, *P* <0.001; Fig 2).

6.5 Discussion

Our results show that sex allocation patterns in *Amphylaeus morosus* vary strongly depending on the presence of a nest guard. The population-wide investment sex ratio was only slightly male-biased. However, the mean sex ratio of social colonies was heavily male-biased, which contrasts with multiple studies on eusocial Hymenoptera that report female-biased allocation associated with worker control over brood rearing (Boomsma 1991; Trivers and Hare 1976).

Why are a set number of females provisioned first in solitary and social nests?

We found that, on average, mothers did not invest differently into individual brood between solitary and social colonies of *A. morosus*. However, we caveat our use of 'investment' to only include the allocation of food resources, where in fact investment into nest construction (Ostwald et al. 2021) and provisioning efficiency (Stevens et al. 2007) paint a more complex picture as these factors are likely to vary dramatically between independent and socially nesting mothers (*SI Appendix*).

Brood sex placement in nests was non-random. In social nests, *A. morosus* exhibits protogyny, whereby females are provisioned in the first series of cells (cells 1-3), followed by predominantly males in the subsequent cell positions (Table 2). Stark (1992) found similar patterns in sex allocation in the carpenter bee *Xylocopa sulcatipes*, which also provisions sequential brood cells in a linear nest. In this bivoltine species, Stark (1992) showed that females are oviposited first, followed by males, suggesting that early provisioning of females allows daughters to assist mothers with guarding and foraging duties when rearing the subsequent brood. Furthermore, female helpers in *X. sulcatipes* do not bias help towards one sex, meaning split sex ratios would not be selected for and would not facilitate queen and worker-like roles. However, this explanation does not hold for *A. morosus* given that it is strictly univoltine and newly emerged females only have an opportunity to guard if they help their mother rear offspring in the following season (Hearn et al. 2022).

The sequence effect of offspring production in *A. morosus* is perhaps better explained by a combination of extrinsic and developmental factors. Females were the slightly larger sex and on average took longer to develop from egg to adult (Hearn pers. obs.). *Amphylaeus morosus* mothers might therefore produce the sex with the longest development time first to avoid subsequent brood cells being trampled upon brood maturation (Bosch and Vicens 2002). Another key determinant could be how quickly mothers can start provisioning, which may determine what sex are laid first (*SI Appendix*). For instance, while floral resources are abundant during the earlier stages of the season, mothers should invest in the more valuable and larger sex (i.e. females) and then switch to the smaller sex (i.e. males) to ensure the greatest return on investment (Charnov et al. 1981; Stevens et al. 2007; Torchio and Tepedino 1980; West 2009). If mothers have a guard defending the nest during provisioning trips they are able to produce more offspring without leaving the nest vulnerable to invaders (Goodell 2003).

For *A. morosus*, social mothers always lay female offspring first, with a mode of three and a maximum of five, followed by a switch to all-male production. This pattern may appear puzzling but concords strongly with the “Constant Philopater” hypothesis (CPH; Rodrigues and Gardner 2016). CPH predicts that a constant number of the more-philopatric sex, in our case females, are produced regardless of maternal condition or the total brood number of brood, to reduce competition between philopatric kin and to avoid underinvestment if resources later become scarce (Rodrigues and Gardner 2016).

Does the presence of a nest guard influence sex ratios in a facultatively social population?

Brood production in solitary nests of *A. morosus* was, on average, female-biased, with solitary foundresses often producing only daughters. Conversely, colonies containing a nest guard invested in female production early into the reproductive season, like solitary nesting females, but then switched into the production of males, resulting in exclusively male-biased broods when a nest guard was present for the entirety of the reproductive season.

Split sex ratios in populations containing both solitary and social colonies may help facilitate evolution of eusociality (Gardner et al. 2012; Smith et al. 2019). In the facultatively social bee, *Megalopta genalis*, sex ratios were biased in accordance with the inclusive fitness predictions for worker control, with female-biased brood in social nests favouring worker-controlled investment. In this situation, workers were able to access provisioned brood cells during the rearing phase, but were never explicitly seen to manipulate queen sex ratios (Smith et al. 2019). Split sex ratios that arise from worker control over offspring sex ratios via preferential feeding or male infanticide (Meunier et al. 2008) is very different from what might be happening with *A. morosus*. For many eusocial species, sex biased ratios can be driven by worker control, leading to queen-worker conflict, whereas in *A. morosus*, guards are not able to directly control sex ratios – their effect is vicarious by enabling queens to simply produce more offspring, where additional offspring are mostly or entirely sons.

What determines population-wide investment ratios when individual strategies deviate from parity?

Fisherian principles predict equal investment in males and females, at a population level, but allows individual strategies to deviate from parity (Fisher 1930). In *A. morosus*, total investment into males and females is approximately equal, which partially supports this prediction. Our results show that both social and solitary mothers produce similar numbers of daughters, meaning that guards, who ultimately influence split sex ratios, vicariously drive the population-wide sex ratio through their decision to be a guard or not. Social mothers have control over the absolute number of daughters produced, so variation in sex ratios ultimately becomes a function of whether females residing in a natal nest decide to guard or else become solitary foundresses.

Interestingly, the strategy to remain in the natal nest as a guard is relatively rare in the population, with only 7% of nests containing guards. It is likely that our sampling effort underestimates the number of social nests in the population, due to mortality of queens prior to nest collections (*SI Appendix*). However, when we considered all highly productive nests (>7 brood cells) to be social, we found no difference in sex allocation between putative social nests and sampled social nests (*SI Appendix*). From a sex allocation viewpoint, guarding as a strategy becomes less desirable, the more guards there are in the population. If nest guards bias population sex ratios towards males, then the social mother should compensate by producing more daughters so that population ratios align back to parity. However, this subsequently suggests that any extra males produced due to having a nest guard begins at a later cell position, and the same declining rewards for guard-enhancement of brood production will persist after that cell position.

Concluding remarks

Amphylaeus morosus presents a model of social evolution that acts as an impediment to eusociality and directly contrasts how split sex ratios have been previously perceived (da Silva 2022; Meunier et al. 2008). Male-biased broods in social colonies arise from the benefit of having a designated nest guard that allows provisioning mothers to increase their offspring production, but where producing more of the larger sex (females) is constrained by time and resources. But this increase in male offspring production reduces the payoffs for guarding behaviour as guards become more common in a population because the reproductive value of males declines as the number of males increases. This effect may put a ‘selective brake’ on the frequency of females opting to take on guarding roles and limit the potential for guarding behaviour to spread. This might help explain why guarding behaviour in *A. morosus* is consistent with inclusive fitness theory but is nevertheless uncommon (Hearn et al. 2022).

6.6 Acknowledgements

We thank Olivia Davies, Tyla Cousins and Georgia Macaulay for assistance with fieldwork. This project was supported by the Australian Federal Governments Research Training Program Scholarship and the Holsworth Wildlife Research Endowment & The Ecological Society of Australia grants awarded to L.R.H. and O.K.D. Nests were obtained under a Victorian National Parks scientific collection permit 10008943.

6.7 References

Alpedrinha J, Gardner A, West SA (2014) Haplodiploidy and the evolution of eusociality: worker revolution. *The American Naturalist*. 184:303–317.

Boomsma JJ (1991) Adaptive colony sex ratios in primitively eusocial bees. *Trends in Ecology & Evolution*. 6:92–95.

Boomsma JJ, Grafen A (1991) Colony-level sex ratio selection in the eusocial. *Hymenoptera Journal of Evolutionary Biology*. 4:383–407.

Bosch J, Vicens N (2002) Body size as an estimator of production costs in a solitary bee. *Ecological Entomology*. 27:129–137.

Charnov EL, Los-den Hartogh RL, Jones WT, van den Assem J (1981) Sex ratio evolution in a variable environment. *Nature*. 289:27–33.

Clutton-Brock TH (1982) Sons and daughters. *Nature*. 298:11–13.

da Silva J (2022) Gene dynamics of haplodiploidy favour eusociality in the Hymenoptera. *Evolution*. In press.

Fisher RA (1930) The genetical theory of natural selection. The genetical theory of natural selection. Clarendon Press, Oxford, England.

Frank SA, Crespi BJ (1989) Synergism between sib-rearing and sex ratio in Hymenoptera. *Behavioral Ecology and Sociobiology*. 24:155–162.

Gardner A, Alpedrinha J, West SA (2012) Haplodiploidy and the evolution of eusociality: split sex ratios. *The American Naturalist*. 179:240–256.

Goodell K (2003) Food availability affects Osmia pumila (Hymenoptera: Megachilidae) foraging, reproduction, and brood parasitism. *Oecologia*. 134:518–527.

Grafen A (1986) Split sex ratios and the evolutionary origins of eusociality. *Journal of Theoretical Biology*. 122:95–121.

Hamilton WD (1964a) The genetical evolution of social behvaiour I. *Journal of Theoretical Biology*. 7:1–16.

Hamilton WD (1964b) The genetical evolution of social behvaiour II. *Journal of Theoretical Biology*. 7:17–52.

Hamilton WD (1967) Extraordinary sex ratios. *Science*. 156:477–488.

Hamilton WD (1972) Altruism and related phenomena, mainly in social insects. *Annual Review of Ecology and Systematics* 3:193–232.

Hearn LR, Davies OK, Schwarz MP (2022). Extreme reproductive skew at the dawn of sociality is consistent with inclusive fitness theory but problematic for routes to eusociality. *Proceedings of the Royal Society B*. 20220652.

Hearn LR, Parslow BA, Stevens MI, Schwarz MP (2022) Temporal dissonance between group size and its benefits requires whole-of-lifecycle measurements. *Behavioural Ecology*. 33:606–614.

IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp.

Kümmerli R, Keller L (2009) Patterns of split sex ratio in ants have multiple evolutionary causes based on different within-colony conflicts. *Biology letters*. 5:713–716.

MacArthur RH (1965) Ecological consequences of natural selection. *Theoretical and Mathematical Biology*. 388–397.

Meunier J, West SA, Chapuisat M (2008) Split sex ratios in the social Hymenoptera: a meta-analysis. *Behavioral Ecology*. 19:382–390.

Mueller UG (1991) Haplodiploidy and the evolution of facultative sex ratios in a primitively eusocial bee. *Science*. 254:442–444.

Ostwald MM, Fox TP, Harrison JF, Fewell JH (2021) Social consequences of energetically costly nest construction in a facultatively social bee. *Proceedings of the Royal Society B: Biological Sciences*. 288:20210033.

R Core Team (2018) *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Rautiala P, Helanterä H, Puurtinen M (2019) Extended haplodiploidy hypothesis. *Evolution Letters*. 3:263–270.

Rodrigues AMM, Gardner A (2016) The constant philopater hypothesis: a new life history invariant for dispersal evolution. *Journal of Evolutionary Biology*. 29:153–166.

Rosset H, Chapuisat M (2006) Sex allocation conflict in ants: when the queen rules. *Current Biology*. 16:328–331.

Seidelmann K (1999) The function of the vestibulum in nests of a solitary stem-nesting bee, *Osmia rufa* (L.). *Apidologie*. 30:19–29.

Smith AR, Kapheim KM, Kingwell CJ, Wcislo WT (2019) A split sex ratio in solitary and social nests of a facultatively social bee. *Biology Letters*. 15:20180740.

Spessa A, Schwarz MP, Adams M (2000) Sociality in *Amphylaeus morosus*. *Annals of the Entomological Society of America*. 93:684–692.

Stark RE (1992) Sex ratio and maternal investment in the multivoltine large carpenter bee *Xylocopa sulcatipes* (Apoidea: Anthophoridae). *Ecological Entomology*. 17:160–166.

Stevens MI, Hogendoorn K, Schwarz MP (2007) Evolution of sociality by natural selection on variances in reproductive fitness: evidence from a social bee. *BMC Evolutionary Biology*. 7:153.

Tepedino VJ, McDonald LL, Rothwell R (1979) Defense against parasitization in mud-nesting Hymenoptera: Can empty cells increase net reproductive output? *Behavioral Ecology and Sociobiology*. 6:99–104.

Torchio PF, Tepedino VJ (1980) Sex ratio, body size and seasonality in a solitary bee, *Osmia lignaria propinqua* Cresson (Hymenoptera: Megachilidae). *Evolution*. 34:993–1003.

Trivers RL, Hare H (1976) Haplodiploidy and the evolution of social insects. *Science*. 191:249–263.

Trivers RL, Willard DE (1973) Natural selection of parental ability to vary the sex ratio of offspring. *Science*. 179:90–92.

West S (2009) Sex allocation. Princeton University Press, Princeton, NJ.

Table 1. Split sex ratios and productivity of *Amphylaeus morosus* solitary and social colonies. 95% CI's for mean numerical sex ratio (NSR) are bootstrapped over 2000 pseudo replicates.

	Nest type	**Sample size (nests)**	**Mean clutch size**	**Mean pupal weight (mg)**		**Mean NSR**			
				Female	**Male**	**Arithmetic**	**95% CI**	**Arcsine back transformed**	**95% CI**
Social status	Solitary	180	5.14 ± 0.219	61.15 ± 0.669	54.19 ± 0.791	0.338	0.282 – 0.395	0.241	0.174 – 0.316
	Social	13	12.2 ± 0.725	58.15 ± 2.99	57.62 ± 1.39	0.834	0.785 – 0.879	0.850	0.791 – 0.905
Population	Mixed	193	5.49 ± 0.229	61.31 ± 0.65	55.05 ± 0.69	0.568	0.536 – 0.599	0.606	0.557 – 653

Table 2. Sex allocation of *Amphylaeus morosus* offspring across cell positions for solitary and social colonies. Deviations from a hypothesised 0.5 sex ratio are shown with a chi-square goodness of fit test.

Brood cell position	**Solitary**					**Social**				
	Total no. of brood	**No. of females**	**Offspring NSR**	χ^2	***P* value**	**Total no. of brood**	**No. of females**	**Offspring NSR**	χ^2	***P* value**
1	119	112	0.058 ± 0.022	92.65	<0.001	11	10	0.091 ± 0.091	7.36	0.007
2	123	114	0.073 ± 0.024	89.63	<0.001	11	8	0.273 ± 0.141	2.27	0.132
3	114	83	0.272 ± 0.042	23.72	<0.001	13	6	0.539 ± 0.142	0.08	0.782
4	97	41	0.577 ± 0.050	2.32	0.128	13	2	0.846 ± 0.104	6.23	0.013
5	61	14	0.754 ± 0.056	15.75	<0.001	13	0	1.00	-	-
6	39	0	1.00	-	-	14	0	1.00	-	-
7	38	1	0.974 ± 0.026	34.11	<0.001	12	0	1.00	-	-
8 – 17	167	0	1.00	-	-	63	0	1.00	-	-

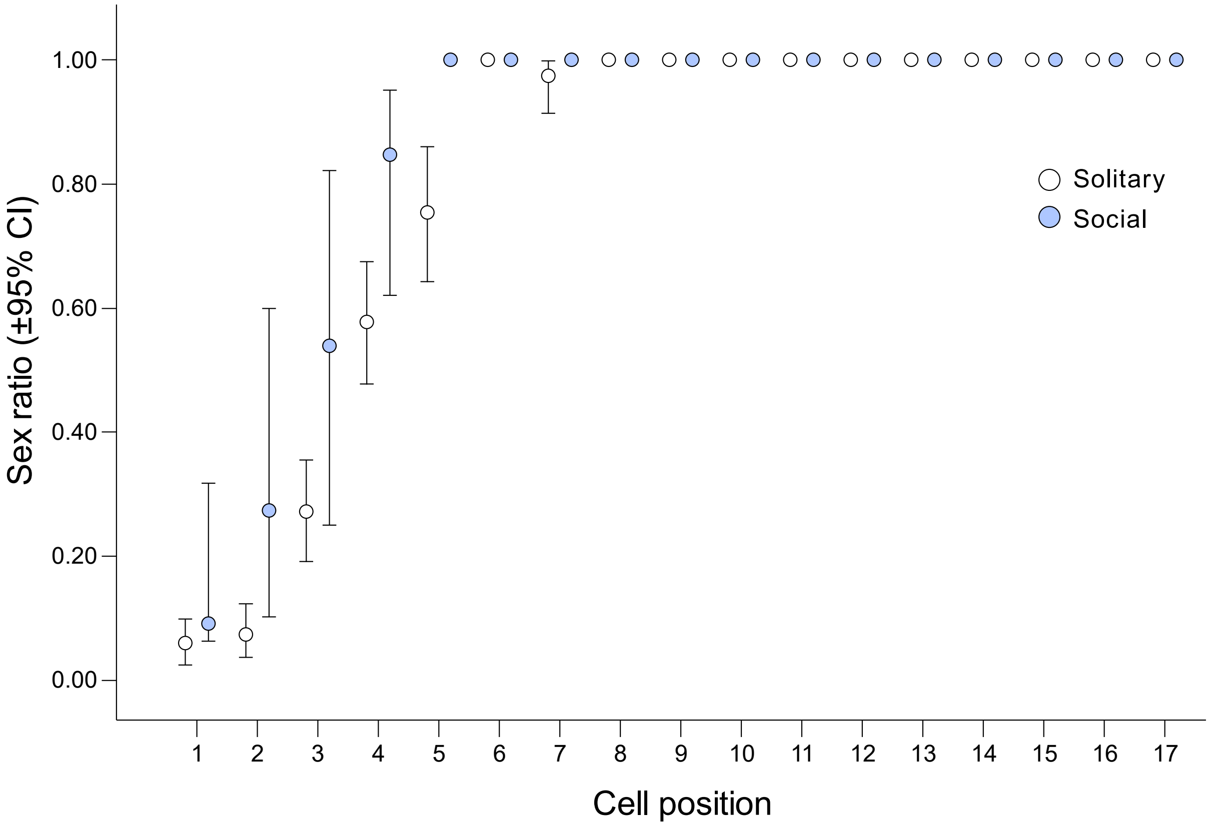

Figure 1. Comparison of operational sex ratios across nest cell positions for solitary and social nests of *Amphylaeus morosus*. Error bars are presented as 95% confidence intervals estimated from 2000 bootstrap pseudo-replicates.

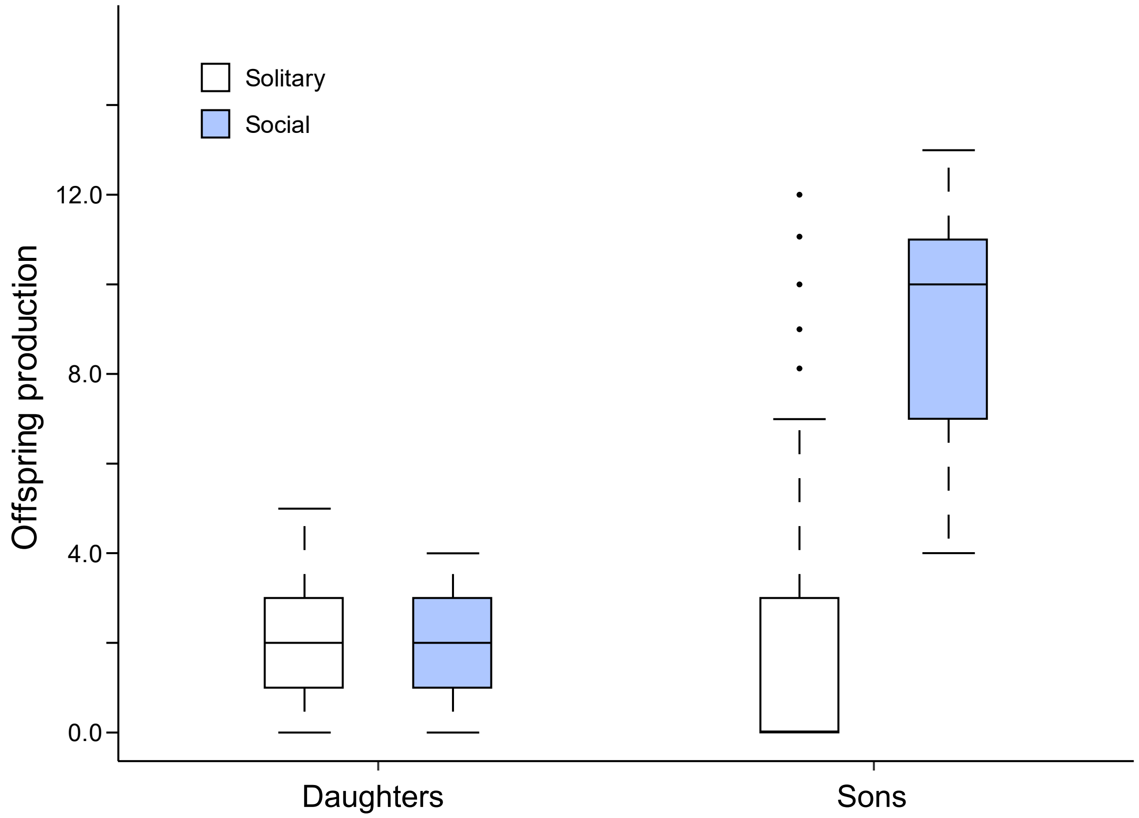

Figure 2. Box plots showing the constant production of daughters and the skewed production of sons across both solitary and social *Amphylaeus morosus* nests.

6.8 Supplementary material

Appendix

Putative social nests

It is possible that nests containing one adult female initially began as social nests, but had lost the primary foraging female due to abandonment or premature mortality prior to collection. While it is impossible to recapture the number of females a nest once had, we can make assumptions based on a number of key variables that distinguish social colonies from solitary nests to reduce this potentially confounding factor.

We used a discriminate analysis to model the probability of nests using six key variables that may predict how many group members a nest once had (variables: clutch size, offspring survival rate, presence of parasitised cells, presence of vestibular cells and the number of male brood and female brood; Table S1). We use this model to determine if the underestimation of social nests accounted for variation in sex ratio across both solitary and social nests. Our stepwise approach determined that a large clutch size (>7 brood cells) was a key determinant for predicting social nests within the sampled population, followed by the number of male brood (Fig. S2). The discriminate analysis predicted 24 (12.4% of total viable nests) solitary nests that were once social (Table S5). When comparing the predicted solitary and social nests from the discriminate analysis with the known sampled solitary and social nests, the patterns in sex ratio did not change (Table S6).

Table S1. List of variables used in the discriminate analyses using a stepwise approach.

Colony type		Mean	Std. Deviation	Valid N (listwise)	
				Unweighted	Weighted
Solitary	Total no. brood cells	5.1206	3.65501	282	282.000
	No. surviving brood cells	2.7447	3.37017	282	282.000
	Proportion surviving brood	.4704	.40047	282	282.000
	False cell	.6596	.47469	282	282.000
	no. female brood	1.3369	1.45269	282	282.000
	no. male brood	1.4113	2.89329	282	282.000
	Parasitised	.3085	.46270	282	282.000
Social	Total no. brood cells	12.3125	2.75000	16	16.000
	No. surviving brood cells	9.5000	4.78888	16	16.000
	Proportion surviving brood	.7786	.33883	16	16.000
	False cell	.0000	.00000	16	16.000
	no. female brood	1.6250	1.20416	16	16.000
	no. male brood	7.8750	3.94757	16	16.000
	Parasitised	.1250	.34157	16	16.000
Total	Total no. brood cells	5.5067	3.95704	298	298.000
	No. surviving brood cells	3.1074	3.77238	298	298.000
	Proportion surviving brood	.4870	.40296	298	298.000
	False cell	.6242	.48515	298	298.000
	no. female brood	1.3523	1.44017	298	298.000
	no. male brood	1.7584	3.29197	298	298.000
	Parasitised	.2987	.45844	298	298.000

Table S2. Variables entered in the discriminate analysis using a stepwise approach.

Step	Entered	Wilks' Lambda							
		Statistic	df1	df2	df3	Exact F			
						Statistic	df1	df2	Sig.
1	no. male brood	.803	1	1	296.000	72.405	1	296.000	.000
2	Total no. brood cells	.792	2	1	296.000	38.629	2	295.000	.000

Table S3. Variables retained in the discriminate analysis using a stepwise approach.

Step		Tolerance	F to Remove	Wilks' Lambda
1	no. male brood	1.000	72.405	
2	no. male brood	.471	14.570	.832
	Total no. brood cells	.471	4.097	.803

Table S4. Variables removed from the discriminate analyses using a stepwise approach.

Step		Tolerance	Min. Tolerance	F to Enter	Wilks' Lambda
0	Total no. brood cells	1.000	1.000	59.941	.832
	No. surviving brood cells	1.000	1.000	57.846	.837
	Proportion surviving brood	1.000	1.000	9.096	.970
	False cell	1.000	1.000	30.792	.906
	no. fem brood	1.000	1.000	.605	.998
	no. male brood	1.000	1.000	72.405	.803
	Parasitised	1.000	1.000	2.438	.992
1	Total no. brood cells	.471	.471	4.097	.792
	No. surviving brood cells	.169	.169	.109	.803
	Proportion surviving brood	.785	.785	.882	.801
	False cell	.764	.764	2.103	.798
	no. fem brood	.980	.980	.147	.803
	Parasitised	.982	.982	.146	.803
2	No. surviving brood cells	.168	.150	.256	.792
	Proportion surviving brood	.755	.374	.305	.792
	False cell	.721	.444	.978	.790
	no. fem brood	.972	.467	.321	.792
	Parasitised	.947	.446	.592	.791

Table S5. Classification table for predicted social and solitary nests from the discriminate analyses.

		Colony type	**Predicted Group Membership**		**Total**
			Solitary	**Social**	
Original	Count	Solitary	268	14	282
		Social	8	8	16
	%	Solitary	95.0	5.0	100.0
		Social	50.0	50.0	100.0
Cross-validated	Count	Solitary	266	16	282
		Social	8	8	16
	%	Solitary	94.3	5.7	100.0
		Social	50.0	50.0	100.0

Table S6. Frequency and sex ratio of putative social and solitary nests from the discriminate analyses and sampled solitary and social nests. Putative social nests represent the total nests classed as social from the discriminate analyses combined with any sampled social nests the discriminate analyses classed as solitary.

	Solitary nests	**Putative solitary nests**	**Social nest**	**Putative Social nests**
Sample size	180	165	13	28
Sex ratio	0.348 ± 0.039	0.298 ± 0.029	0.834 ± 0.024	0.827 ± 0.014

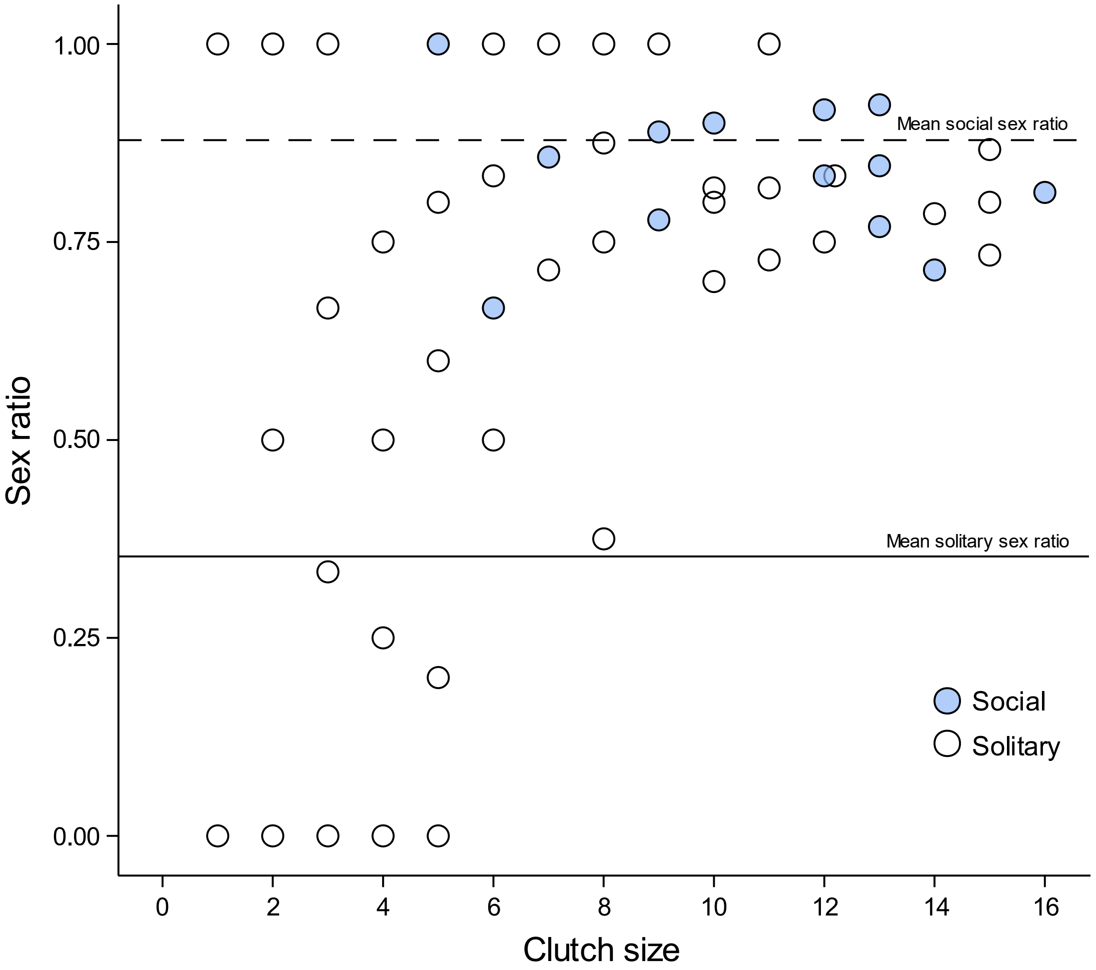

Figure S1. Sex ratio of *Amphylaeus morosus* progeny in social and solitary nests. Lines represent the population numerical sex ratios for social and solitary nests. Sex ratio varied significantly with clutch size ($F_{1,\ 192}$ = 106.3, r = 0.358, P <0.001).

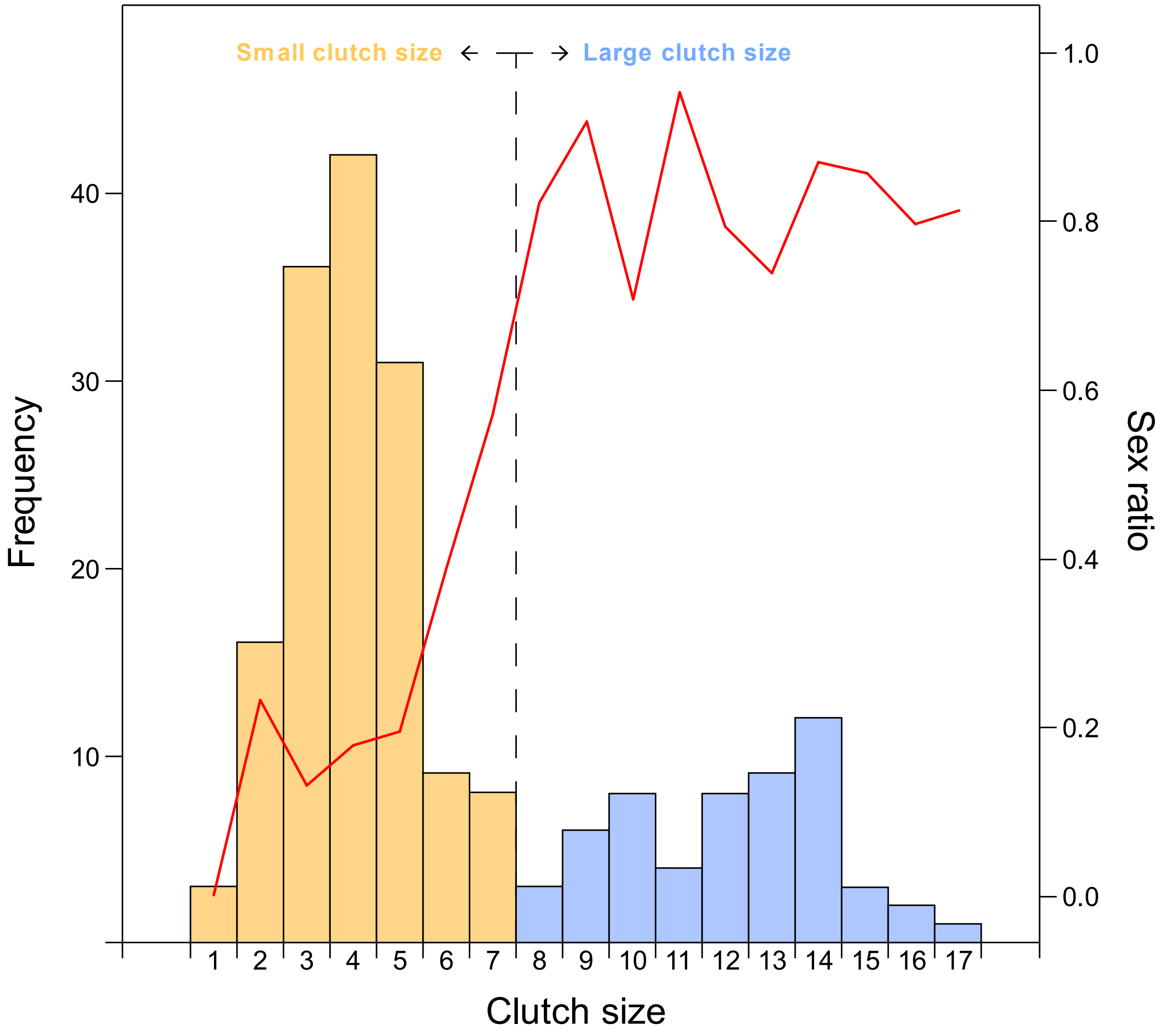

Figure S2. Frequency distribution of offspring production within nests showing the approximate division of nests according to the two key predictor variables of the discriminate analysis (clutch size and male brood production).

Factors contributing to sex ratio patterns

Below I highlight some factors that may contribute to sex investment ratio patterns in *A. morosus*.

Role of the vestibulum and parasitism

Nest architecture in this species is dichotomous based on the presence or absence of vestibular cells interspersing brood cells (Hearn et al. 2021). Vestibular cells are empty chambers that may be positioned throughout nests and are thought to act as a strategy to deter parasites (Seidelmann 1999; Tepedino et al. 1979). In *A. morosus*, vestibular structures are only employed in solitary nests, while nests with sequential brood cells may be either solitary or social (Hearn et al. 2021).

Distinct patterns in sex ratio were observed across different nest structures produced by *A. morosus* females. Nests containing at least one vestibular cell were heavily female-biased (at least one vestibulum: NSR = 0.176) and nests containing no vestibules male-biased (no vestibulum: NSR = 0.763; Table S7).

The variability of sex allocation patterns throughout the study population indicates the potential influence of extrinsic factors that may result in adaptive sex ratios. Environmental conditions have been shown to effect colony sex ratios in numerous aculeate Hymenoptera. In *A. morosus*, numerical sex ratios were markedly different between nests containing vestibular cells and nests where brood cells were laid sequentially (Table S7). It is worth noting that these patterns which coincide with the split sex ratios seen across social polymorphisms could be a factor of solitary nests primarily containing at least one vestibular cell and social nests always having sequential brood cells.

Table S7. Comparison of productivity and sex ratios across nests containing at least one vestibular cell and nests with sequentially laid brood cells.

Nest type	**Sample size (nests)**	**Mean clutch size**	**NSR**
Vestibulum	125	4.01 ± 0.122	0.176 ± 0.026
Sequential	68	10.49 ± 0.471	0.763 ± 0.031

Seasonal and aseasonal patterns in sex allocation

Sex ratio became significantly more female-biased as the reproductive season progressed (Kruskal-Wallis: H = 7.889, d.f. = 2, P = 0.019; Fig S3). Sex ratios for social colonies remained consistently male-biased across years compared to solitary nests which remained female-biased across sampling years (Table S9). No social nests were collected during the later periods of the reproductive season (February). This is most likely due to the increased chance of the primary foraging and reproductive female dying prior to nest collection (Table S9).

Table S8. Distribution of all solitary and social nests collected for each sampling period.

Sample	**Colony type**		**Total**
	Solitary	**Social**	
1	78	3	81
2	6	0	6
3	23	2	25
3a	25	0	25
3b	13	0	13
4	63	8	71
4a	74	3	77
Total	282	16	298

Table S9. Sex ratios for nests collected across consecutive reproductive seasons (years) and nests collected at each month within a reproductive season. Collections from november have been excluded due higher than expected nest failure rates prior to nest opening.

Colony type	**Year**							
	2017		**2018**		**2019**		**2020**	
	N	**NSR**	***N***	**NSR**	***N***	**NSR**	***N***	**NSR**
Solitary	51	0.291 ± 0.055	4	0.500 ± 0.289	28	0.149 ± 0.060	94	0.415 ± 0.040
Social	3	0.838 ± 0.036	0	n/a	0	n/a	11	0.833 ± 0.029

	Month					
	December		**January**		**February**	
	N	**NSR**	***N***	**NSR**	***N***	**NSR**
Solitary	99	0.404 ± 0.041	70	0.272 ± 0.044	8	0.129 ± 0.098
Social	11	0.818 ± 0.026	3	0.893 ± 0.054	0	n/a

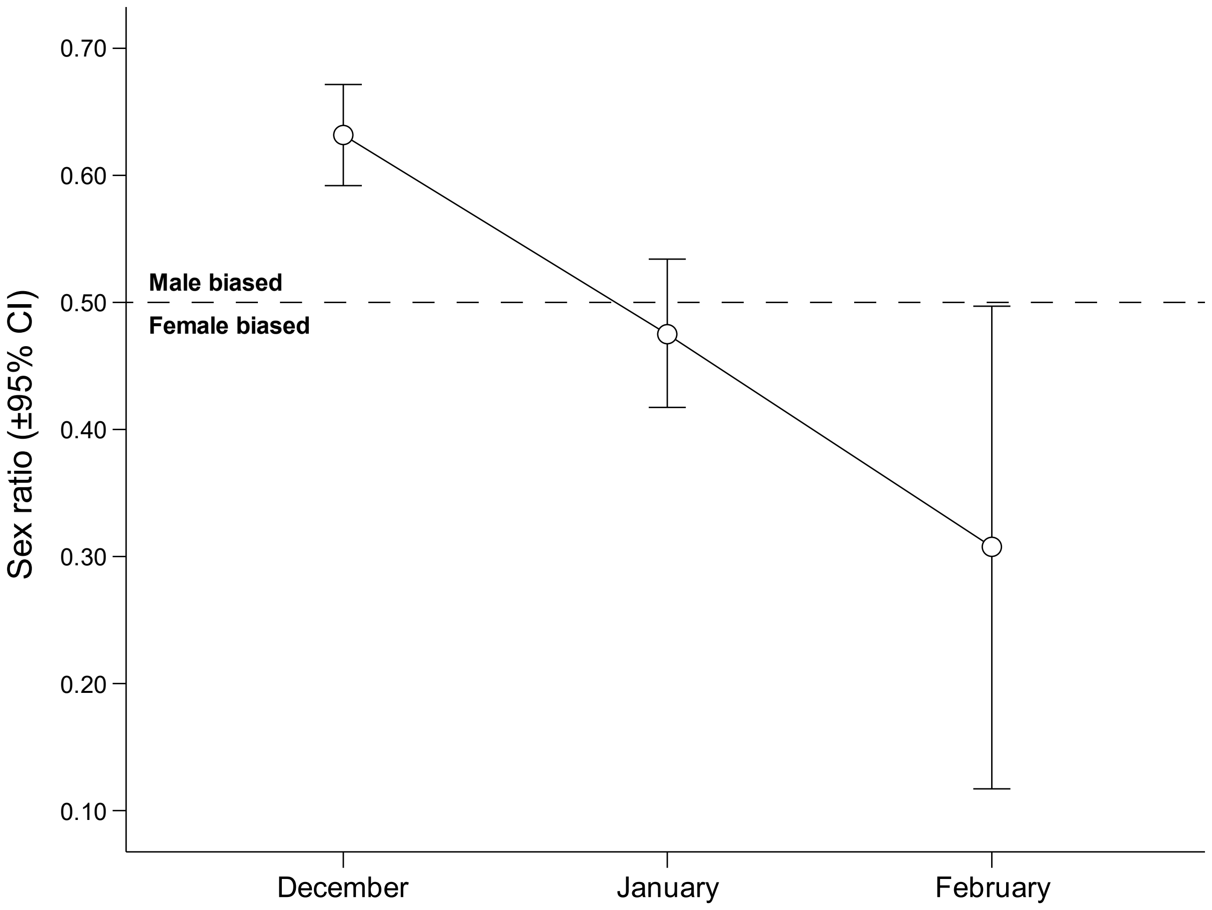

Figure S3. Mean numerical sex ratio of all viable nests across the reproductive season.

Provisioning rate

The abundance of floral resources in the environment is an important factor that can determine how mothers invest into each sex. While we were unable to track floral availability, we can ask how quickly females re-using a natal nest or constructing a new nest are able to start provisioning based on offspring production throughout the reproductive season.

There was no difference in offspring production for females re-using a natal nest but a significant difference for females constructing a new nest across the reproductive season (Re-used: Kruskal-Wallis: H = 4.830, d.f. = 2, P = 0.185; New: Kruskal-Wallis: H = 12.785, d.f. = 2, P = 0.005; Fig S4). Early in the brood provisioning and production phase (December) the mean number of offspring produced was significantly greater in re-used nests compared to newly constructed nests (Re-used = 7.36 ± 0.474, New = 3.35 ± 0.551; Mann-Whitney: U = 644.0, P <0.001), suggesting females re-using a natal nest are able to start provisioning earlier.

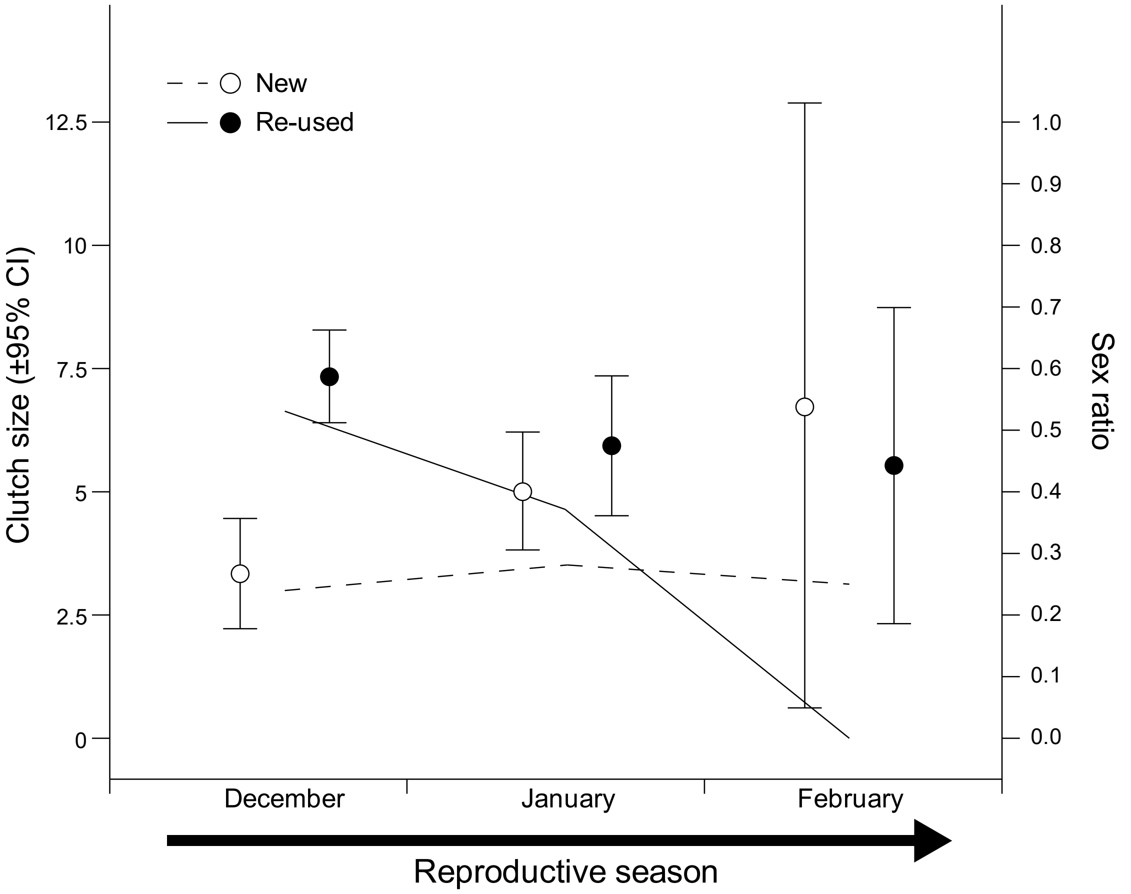

Figure S4. Mean offspring produced (error bars) and numerical sex ratio (lines) for re-used natal nests or newly constructed nests.

References

Hearn LR, Stevens MI, Schwarz MP, Parslow BA (2021) Parasitoids of the uniquely social colletid bee *Amphylaeus morosus* (Hymenoptera: Colletidae) in Victoria Memoirs of Museum Victoria 80:183–191.

Seidelmann K (2006) Open-cell parasitism shapes maternal investment patterns in the Red Mason bee *Osmia rufa*. *Behavioral Ecology*. 17:839–848.

Tepedino VJ, McDonald LL, Rothwell R (1979) Defense against parasitization in mud-nesting Hymenoptera: Can empty cells increase net reproductive output? Behavioral Ecology and Sociobiology 6:99–104.

General discussion

This thesis aimed to understand the various factors that promote social nesting in the only known social colletid bee species, *Amphylaeus morosus*, and thus gain insights into the very earliest stages of insect social evolution.

The key paradigms of social evolution

A key goal of sociobiology is to understand the selective forces that allow for reproductive altruism to evolve. A lot of attention has been directed at highly derived eusocial forms characterised by worker sterility, but exactly how cooperative nesting initially evolves from solitary living is still poorly understood (Rehan and Toth 2015; Szathmáry and Smith 1995). Theoretical explanations for the formation of social groups, such as kin selection theory, whereby genetic relatives gain indirect fitness through helping kin, currently provide the meain paradigm for untangling the paradoxical behaviour of reproductive altruism (Hamilton 1964*a,b*). However, a lack of empirical support combined with recent evolutionary models that tend to diminish the importance of within-group relatedness has questioned the primacy of kin selection, with many recent studies opting for alternative explanations for cooperative behaviour in empirical systems (Gadagkar 2016; Kukuk et al. 2005; Ohkubo et al. 2018; Ostwald et al. 2021; Stevens et al. 2007; Vickruck and Richards 2021; Yagi and Hasegawa, 2012). In chapter 1, I proposed five alternative models that may be operating in conjunction with kin selection in insect species, listing some early forms of social behaviour where kin selection has been questioned as having a key role.

Numerous insect species fall into the scope of chapter 1's review where social nesting seemingly requires explanations other than kin selection. However, as I have shown through the progession and development of this thesis, *A. morosus* presents one of the few species where kin selection reckonings align with social nesting at an early stage of social evolution and does not fall into the scope of my review. The discrepancy in the outcomes of chapter 1 and chapter 5 also highlights the need to re-evaluate relatedness estimates calculated from allozymes using next generation sequencing approaches that might reveal more accurate relatedness estimates in support of kin selection.

Ecological factors contributing to sociality

Detailed life-history traits of social hosts and their parasites are generally poorly understood for many social Hymenoptera and can lead to inaccurate conclusions regarding the role of parasites in driving social behaviour. In chapters 2 and 3 I described the host-parasitoid associations for eight different parasitoid species attacking *A. morosus* nests in the Dandenong Ranges, Victoria, Australia. I found that parasitoid phenologies strongly differed from their host and between parasitoid species themselves. I found that there was a chronological order of oviposition from each parasitoid species, with gasteruptiid wasps at the start of the reproductive phase, *Anthrax maculatus* in the middle and both mutillid wasp species attacking towards the end of the reproductive phase. In chapter 4 I investigated these various parasite-mediated selection pressures inflicted on *A. morosus* throughout the reproductive phase. I showed that the eight different parasitoid species tended to attack their host at different times over the reproductive season, with a heightened risk of parasitism towards the end of the season from mutillid wasps. These pressures across the reproductive season create a disconnect between the apparent benefits gained early in the season when parasite pressure is low, and the benefits gained later in the season from having a defender remaining in the nest. The problem arising from these delayed benefits of group size was termed 'temporal dissonance' and has significant implications for understanding how social dynamics change throughout a life-cycle while highlighting the importance of targeted sampling methods that take a 'whole-of-life-cycle' approach.

Further to this, my findings in chapter 5 show that in *A. morosus* there is a significant advantage in having at least one adult female remaining in the nest until offspring have reached adulthood (Hearn et al. 2022). Adult presence in the nest can provide defence against parasitism and predation (e.g. Smith et al. 2003) and higher overall reproductive output and brood survivorship. Comparisons of productivity between social and solitary nests in chapter 5 revealed significant benefits to social nesting. On average, the productivity of social nests increased twofold, compared to solitary nests, and they were able to better defend against nest parasites. The importance of nest defence has often been hypothesised to select for social living in insects (Crespi 1994; Gadagkar et al. 1990; Lin and Michener 1972; Wilson 1971). Yet, in cases where defence against natural enemies is directly testable between social and solitary polymorphisms, these assumptions have often fallen short (Prager 2014; Smith et al. 2017). My findings showed that *A. morosus* is able to successfully defend against the numerous nest invaders that pressure this species throughout the reproductive period (Hearn et al. 2021), most likely through the employment of a nest guard that is able to physically block the nest entrance and fight off intruders (Hearn pers. obs.). These findings suggest that defence against nest parasitism at the earliest stage of insect sociality promotes for nest guards which could have implications for the evolution of more sophisticated worker castes.

Evolution of haplodiploid workers

In chapter 5 I showed that the temporally variable parasite pressures demonstrated in chapter 4 are likely to translate to the formation of familial associations that can protect against parasitism throughout the brood rearing season. If at least one female is able to remain in the nest until the end of the reproductive season, then it could have significant fitness payoffs for all related nestmates if the nest can be successfully defended until the brood have matured (Gadagkar et al. 1990).

Amphylaeus morosus formed both matrifilial and full-sibling colonies that consisted of one reproductive primary and one secondary female that appeared to sacrifice the reproductive opportunity of nesting alone to stay and help guard the nest from intruders. Interestingly, I found no observable difference in morphological traits aside from wing wear between reproductive primary and secondary females. Secondary females appeared to be unworn, with minimal wear to their wings and body, suggesting that they wait as a nest guard, which could be a low-risk role with few mortality risks unless defence is actually needed, and avoids the high physiological costs associated with foraging (Schwarz et al. 2011; Vickruck and Richards 2018).

Combining genomic and life-history data, I showed that reproductive altruism between the reproductive primary and nest guard in social nests was consistent with Hamilton's inclusive fitness theory (Hamilton 1964a,b). But also, that, despite the asymmetrical relatedness between guard and offspring in matrifilial and full-sibling colonies, the inclusive fitness benefits for a guard female did not vary appreciably across colony type. This approximately equal attribution of benefits for guard females is driven by male-biased broods in social colonies, which distort the genetic relatedness away from the optima for a guard female. If workers are not able to directly enhance sex ratios in their favour, then it suggests that not only can effective sterility in worker castes evolve at the earliest stages of sociality, but also that the previously hypothesised subsocial and semisocial pathways to eusociality (Lin and Michener 1972) show no selective differences that may favour one particular strategy during the initial phases of social evolution.

Factors inhibiting sociality

In chapter 5 I showed that the male-biased broods produced by social *A. morosus* colonies potentially present a selection barrier to more frequent guarding behaviour in populations. Sex biased ratios have been linked to the evolution of sociality as maternal investment patterns in social Hymenoptera can sometimes favour the production of females early in a season (protogyny) and these early daughters can then become workers and assist in rearing future broods that benefit worker and mother alike (Johnstone et al. 2012). In chapter 6 I explored the puzzling sex ratios across solitary and social *A. morosus* nests. I found that male-biased broods in social colonies arise from the benefit of having a nest guard that allows provisioning mothers to increase their offspring production, but where producing more daughters is constrained, possibly via the selective mechanisms outlined in the "Constant Philopater" hypothesis (Rodrigues and Gardner 2016). This increase in male offspring production reduces the payoffs for guarding behaviour as guards become more common in a population because the reproductive value of males declines as the number of males increases. This effect may inhibit the frequency of females opting to take on guarding roles and limit the potential for guarding behaviour to spread. This might help explain why guarding behaviour in *A. morosus* is consistent with inclusive fitness theory, as shown in chapter 5, but is not maintained at a higher frequency within the population.

Conclusion

In summary, this thesis has demonstrated three key findings that both support and challenge previous assumptions relating to the major transition from solitary to social living in insects. Firstly, I have shown that parasitoids can play a role in promoting cooperative nesting in insect species, but to fully understand exactly how they influence social nesting requires that we understand how host and parasites operate across the full life-cycle. Secondly, I have provided empirical evidence that a species entering the first steps into social living exhibits reproductive altruism that is consistent with inclusive fitness theory. Finally, I have demonstrated that male-biased sex ratios in social colonies arise as a condition of receiving help, allowing mothers to provision more brood cells, but subsequently this increase in male brood production lowers the threshold for worker altruism to persist. Combined my findings provide a unique perspective into the major transition from solitary living to complex social organisation in insects and will enable a greater understanding of the earliest stage of insect sociality.

Future directions

While this thesis has explored how social behaviour has initially evolved in *A. morosus*, two important aspects need further consideration to understand how social behaviour is maintained in this species. Firstly, detailed within nest observations are needed to quantify the costs of contests between social nestmates, which are likely to differ between full-sibling and matrifilial colonies depending on the degree of relatedness between nestmates and decipher which female takes on the primary reproductive role. For matrifilial colonies of *A. morosus*, the primary reproductive was always the matriarch and this is a common hierarchal progression in other social Hymenoptera (West-Eberhard 1978). However, the determinant of reproductive dominance is still unclear for full-sibling nests, where all natal daughters theoretically have opportunities to reproduce: we need to understand how the dichotomy of ‘queen’ and ‘guard’ arises and whether it involves some kind of power asymmetry. Second, the ability for adult females to survive into a consecutive year as evidenced by mother-daughter associations needs longitudinal investigation over consecutive years. Studies of longevity can help further untangle the role of the secondary female and how it might persist into a second season. Secondary females appear to passively wait in the nest as a way to preserve longevity and ensure survival until brood have reached maturity in the event that the primary female dies (Schwarz et al. 2011). Subsequently, this could permit secondary females a mechanism to survive into a consecutive season and take the primary reproductive role as has been reported in the large carpenter bee, *Xylocopa virginica* (Vickruck and Richards 2018). Investigating adult senescence could therefore determine whether secondary females in matrifilial colonies are preserving energy that allows them to survive into a consecutive season and this could be tested with the presence of lipofuscins (Robson and Crozier 2009).

References

Crespi B (1994) Three conditions for the evolution of eusociality: are they sufficient? *Insectes Sociaux*. 41:395–400.

Gadagkar R (1990) Evolution of eusociality: the advantage of assured fitness returns. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 329:17–25.

Gadagkar R (2016) Evolution of social behaviour in the primitively eusocial wasp *Ropalidia marginata*: do we need to look beyond kin selection? *Philosophical Transactions of the Royal Society B: Biological Sciences*. 371:20150094.

Hamilton WD (1964*a*) The genetical evolution of social behvaiour I. *Journal of Theoretical Biology*. 7:1–16.

Hamilton WD (1964*b*) The genetical evolution of social behvaiour II. *Journal of Theoretical Biology*. 7:17–52.

Hearn LR, Stevens MI, Schwarz MP, Parslow BA (2021). Parasitoids of the uniquely social colletid bee *Amphylaeus morosus* (Hymenoptera: Colletidae) in Victoria. *Memoirs of Museum Victoria*. 80:183–191.

Hearn LR, Parslow BA, Stevens MI, Schwarz MP (2022). Temporal dissonance between group size and its benefits requires whole-of-lifecycle measurements. *Behavioral Ecology*. 33:606–614.

Johnstone RA, Cant MA, Field J (2012) Sex-biased dispersal, haplodiploidy and the evolution of helping in social insects. *Proceedings of the Royal Society* B. 279:787–793.

Kukuk PF, Bitney C, Forbes SH (2005) Maintaining low intragroup relatedness: evolutionary stability of nonkin social groups. *Animal Behaviour*. 70:1305–1311.

Lin N, Michener CD (1972) Evolution of Sociality in Insects. *The Quarterly Review of Biology*. 47:131–159.

Ohkubo Y, Yamamoto T, Ogusu N, Watanabe S, Murakami Y, Yagi N, Hasegawa E (2018) The benefits of grouping as a main driver of social evolution in a halictine bee. *Science Advances*. 4:e1700741.

Ostwald MM, Dahan RA, Shaffer Z, Fewell JH (2021) Fluid nest membership drives variable relatedness in groups of a facultatively social bee. *Frontiers in Ecology and Evolution*. 9:767380.

Prager SM (2014) Comparison of social and solitary nesting carpenter bees in sympatry reveals no advantage to social nesting. *Biological Journal of the Linnean Society*. 113:998–1010.

Rehan SM, Toth AL (2015) Climbing the social ladder: the molecular evolution of sociality. *Trends Ecology Evolution*. 30:426–433.

Robson SKA, Crozier RH (2009) An evaluation of two biochemical methods of age determination in insects (pteridines and lipofuscins) using the ant *Polyrhachis sexpinosa* Latrielle (Hymenoptera: Formicidae). *Australian Journal of Entomology*. 48:102-106.

Rodrigues AMM, Gardner A (2016) The constant philopater hypothesis: a new life history invariant for dispersal evolution. *Journal of Evolutionary Biology*. 29:153–166.

Schwarz MP, Tierney SM, Rehan SM, Chenoweth LB, Cooper SJ (2011) The evolution of eusociality in allodapine bees: workers began by waiting. *Biology Letters*. 7:277–280.

Smith AR, Harper C, Kapheim K, Simons M, Kingwell C, Wcislo W (2017) Effects of social organization and resource availability on brood parasitism in the facultatively social nocturnal bee *Megalopta genalis*. *Insectes Sociaux*. 65:85–93.

Smith AR, Wcislo WT, O'Donnell S (2003) Assured fitness returns favor sociality in a mass-provisioning sweat bee, *Megalopta genalis* (Hymenoptera: Halictidae). *Behavioral Ecology and Sociobiology*. 54:14–21.

Stevens MI, Hogendoorn K, Schwarz MP (2007) Evolution of sociality by natural selection on variances in reproductive fitness: evidence from a social bee. *BMC Evolutionary Biology*. 7:153.

Szathmáry E, Smith JM (1995) The major evolutionary transitions. *Nature*. 374:227–232.

Vickruck JL, Richards MH (2018) Linear dominance hierarchies and conditional reproductive strategies in a facultatively social carpenter bee. *Insectes Sociaux*. 64:619–629.

Vickruck JL, Richards MH (2021) Competition Drives Group Formation and Reduces Within Nest Relatedness in a Facultatively Social Carpenter Bee. *Frontiers in Ecology and Evolution*. 9:738809.

West-Eberhard MJ (1978). Polygyny and the Evolution of Social Behavior in Wasps. *Journal of the Kansas Entomological Society*. 51:832–856.

Wilson EO (1971) The insect societies. The insect societies. Belknap Press of Harvard University Press.

Yagi N, Hasegawa E (2012) A halictid bee with sympatric solitary and eusocial nests offers evidence for Hamilton's rule. *Nature Communications*. 3: 939.