Interactions between Hymenopteran species associated with gall-forming wasps: the *Leptocybe invasa* community as a case study

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Abstract

1. *Leptocybe invasa* is native to Australia and induces galls on various species of *Eucalyptus*. Two genetically distinct lineages of this wasp have been detected outside its native range, namely *Leptocybe* Lineage A and *Leptocybe* Lineage B.
2. The parasitoid *Selitrichodes neseri* was released in South Africa as a biological control agent against *L. invasa*. Another parasitoid of *L. invasa*, *Quadrastichus mendeli,* as well as *Megastigmus zebrinus* (parasitoid) and *M. pretorianensis* (role unknown), have also been recorded emerging from *L. invasa* galls. The objective of this study was to investigate the interactions between the different hymenopterans associated with *L. invasa* galls in South Africa.
3. *Leptocybe invasa* galls were dissected and species-specific primers and restriction enzymes were used to identify the larvae where interactions were noted.
4. *Selitrichodes neseri*, *Q. mendeli* and *M. zebrinus* were confirmed to parasitize *Leptocybe* Lineage A, and *S. neseri* was confirmed to parasitize *Leptocybe* Lineage B. Furthermore, there were direct interactions between these parasitoids, where parasitoids were found parasitising each other. The gall forming experiment confirmed that *M. pretorianensis* is not a gall former, but other potential roles remain uncertain.

Introduction

Plant galls are a result of atypical growth and abnormal cell differentiation stimulated by a foreign organism (viruses, bacteria, nematodes, fungi or arthropods) (Meyer, 1987; Mani, 1992; Price, 2005; Shorthouse *et al*., 2005; Gullan & Cranston, 2010). These galls are usually highly organized structures, which tend to be specific to a certain species and in some instances can be used to identify the gall former. Gall induction has evolved independently in six different insect orders (Nyman *et al*., 1998; Cśoka *et al*., 2017). The principle gall formers occur in three orders, namely Hemiptera, Diptera and Hymenoptera (Shorthouse *et al*., 2005).

Gall inducing wasps occur in the hymenopteran suborders Symphyta and Apocrita (Meyer, 1987). Symphyta are primarily represented by sawflies (Tenthredinidae) that induce galls on *Salix* spp. (willows). Two subfamilies that cause gall formation, Cynipoidea and Chalcidoidea exist in Apocrita (Meyer, 1987). Cynipoidea are primarily comprised of the gall-inducing Cynipidae, which are commonly referred to as gall wasps or cynipids, with their hosts predominantly in the Fabaceae family (Abe *et al*., 2014; Cśoka *et al*., 2017). Chalcidoidea, also known as chalcids, induce galls on the buds, leaves and growing shoots of plants (Dreger-Jauffret & Shorthouse, 1992; Klein, 2009). Within the Chalcidoidea, the subfamily Eulophidae accounts for approximately 20% of the described species (Gibson *et al*., 1999; Noyes, 1998; Goméz & Nieves-Aldrey, 2017). Gall-inducing eulophids are represented by two groups: *Ophelimini* (Australian lineage) which consist of gall-inducers primarily on eucalypts and other *Myrtaceae* (Bouček, 1977; Kim *et al*., 2004). Tetrastichinae are more commonly known to be entomophagous parasitoids on a variety of arthropod hosts (La Salle, 1994; Kim & La Salle, 2008). In gall inducing Hymenoptera, the entire immature development of the insect, as well as their parasitoids, occurs within the confines of the gall.

The blue gum chalcid, *Leptocybe invasa* (Fisher & LaSalle), is a minute black wasp that causes the formation of round bump-like galls on the midribs, petioles and stems of *Eucalyptus*. The wasp is likely native to Australia (Otieno *et al.* 2019) but has become a pest on *Eucalyptus* outside its native range (Mendel *et al*., 2004; Protasov *et al*., 2007). It was discovered in the Middle East and Mediterranean region in 2000 and has since spread to about 40 countries, including South Africa (Mendel *et al*., 2004; Zheng *et al*., 2014). In high densities these wasps can cause crown dieback, stunted growth and in some cases tree death (Mendel *et al*., 2004; Zheng *et al*., 2014; Nugnes *et al*., 2015; Csóka *et al*., 2017). Application of molecular markers suggested that the global distribution of *L. invasa* is represented by two different lineages that potentially represent different species (Nugnes *et al*., 2015). The original lineage found in 2000, hereafter referred to as Lineage A (mitochondrial haplogroup A), and the

more recent lineage, hereafter referred to as Lineage B (mitochondrial haplogroup B and C) (Dittrich-Schröder *et al*., 2018). Otieno *et al.* (2019) subdivided Lineage A into regional populations (African, Australian, American, Asian and Mediterranean) and found the Mediterranean and Asian populations occupied different niches compared to other populations.

As is common with insect gall formers, there are several other insect members associated with *L. invasa* and its gall. Although gall formers are encapsulated and protected by the plant, they are not entirely free of natural enemies. This includes parasitoids which develop internally or externally on their host (Schönrogge *et al*., 2012). These parasitoids are extremely specialised at gaining access to the gall, and usually have a narrow host range. Galls can also contain inquilines, which are incapable of initiating the development of a gall but are specialised at gaining access to existing galls. Inquilines do not feed on the gall former, but on the nutritive plant tissue of the gall (Brooks & Shorthouse, 1998). The gall former and inquiline can live together, allowing the full development of both species, but competition for resources within the gall can also result in the death of the gall former (Brooks & Shorthouse, 1998; Ferraz & Monteiro, 2003; Klein, 2009).

In South Africa, hymenopterans associated with *L. invasa* and its galls include two biological control agents, namely *Selitrichodes neseri* Kelly & La Salle (Hymenoptera: Eulophidae) and *Quadrastichus mendeli* Kim & La Salle (Hymenoptera: Eulophidae). *Selitrichodes neseri* is a larval ectoparasitoid that was collected in Australia in 2010 (Kelly *et al*., 2012) and released as a biological control agent in South Africa in 2012 (Dittrich-Schröder *et al*., 2014). *Quadrastichus mendeli* is another larval ectoparasitoid of *L. invasa*. This was released as a biological control agent in Israel in 2003 (Kim *et al*., 2008; Zheng *et al*., 2014) and recorded in South Africa in 2017 as an apparent unintentional introduction (Bush *et al*., 2017). The original releases of *S. neseri* and *Q. mendeli* as biological control agents occurred in different countries and before the discovery of Lineage B of *L. invasa*. Whether the parasitism rates of these two parasitoids differs between Lineage A and Lineage B of *L. invasa,* as well as the interaction of the two parasitoids with each other has not been tested.

Two *Megastigmus* species (Hymenoptera: Torymidae), namely *M. zebrinus* and *M. pretorianensis* are associated with *L. invasa* galls in South Africa (Bush *et al*., 2017). The biology of this family is very diverse. Half of the species feed solely on seeds of the Pinaceae and Rosaceae plant species (Scheffer & Grissel, 2003; La Salle, 2005). Other species in this genus include parasitoids, inquilines and gall formers (Grissell, 1999; Protasov *et al*., 2007; Klein 2009). *Megastigmus zebrinus* is thought to be native to Australia, but was detected from galled seed capsules of *Eucalyptus camaldulensis* in the Western Cape, South Africa (Grissell, 2006; Klein, 2009) and later

from galls of *L. invasa*. *Megastigmus zebrinus* was originally thought to be a gall former on the seeds of *Eucalyptus camaldulensis*, but it was later confirmed that *M. zebrinus* is a parasitoid of the gall former *Quadrasticholla nova* (Hymenoptera: Eulophidae) of *Eucalyptus* seed capsules (Klein, 2009). However, it is yet to be established whether *M. zebrinus* parasitizes *L. invasa* and how it interacts with *S. neseri* and *Q. mendeli* is currently unknown. *Megastigmus pretorianensis* was first described from *L. invasa* galls in South Africa (Doĝanlar *et al*., 2015), but more recently reported from Australia (Le *et al.* 2020), where it is likely native. It is not known if *M. pretorianensis* is a gall former, parasitoid or inquiline.

The objective of this study was to characterise the potential roles and interactions of *S. neseri, Q. mendeli, M. zebrinus* and *M. pretorianensis* in the *L. invasa* gall community in South Africa. Molecular methods using restriction enzymes and species-specific primers were used to distinguish between interacting larvae in the same gall. We further tested whether *M. pretorianensis* was a potential gall former by exposing the wasps to eucalypt seedlings.

Materials and methods

Collection and dissection of galled material

Galled *Eucalyptus* plant material of various species/hybrids was collected from Pretoria (Gauteng), Zululand (KwaZulu-Natal), the Midlands (KwaZulu-Natal), Tzaneen (Limpopo), the Highveld (South Mpumalanga) and the Lowveld (North Mpumalanga). A total of 372 galls were dissected under a Nikon SMZ1500 microscope (x20 magnification). Galls of different ages (age based on size and colour: Mendel *et al*., 2004) (Supplementary Material, Table S1) were dissected to increase the chance of encountering each hymenopteran species, because different parasitoids and inquilines may prefer the gall at a certain developmental stage. The leaf tissue was slowly peeled off using a surgical blade until there were signs of the gall cavity. The gall cavity contains the larva for the duration of its development. For gall cavities that contained more than one larva, the type of interaction and life stage of each occupant was noted (larva, pupa, pre-adult, callowed adult) (Figure 1; Figure 2). From these cavities, each larva, pupa, pre-adult or callowed adult was placed in a labelled 0.2 µl PCR tube for DNA extraction and molecular identification as described below.

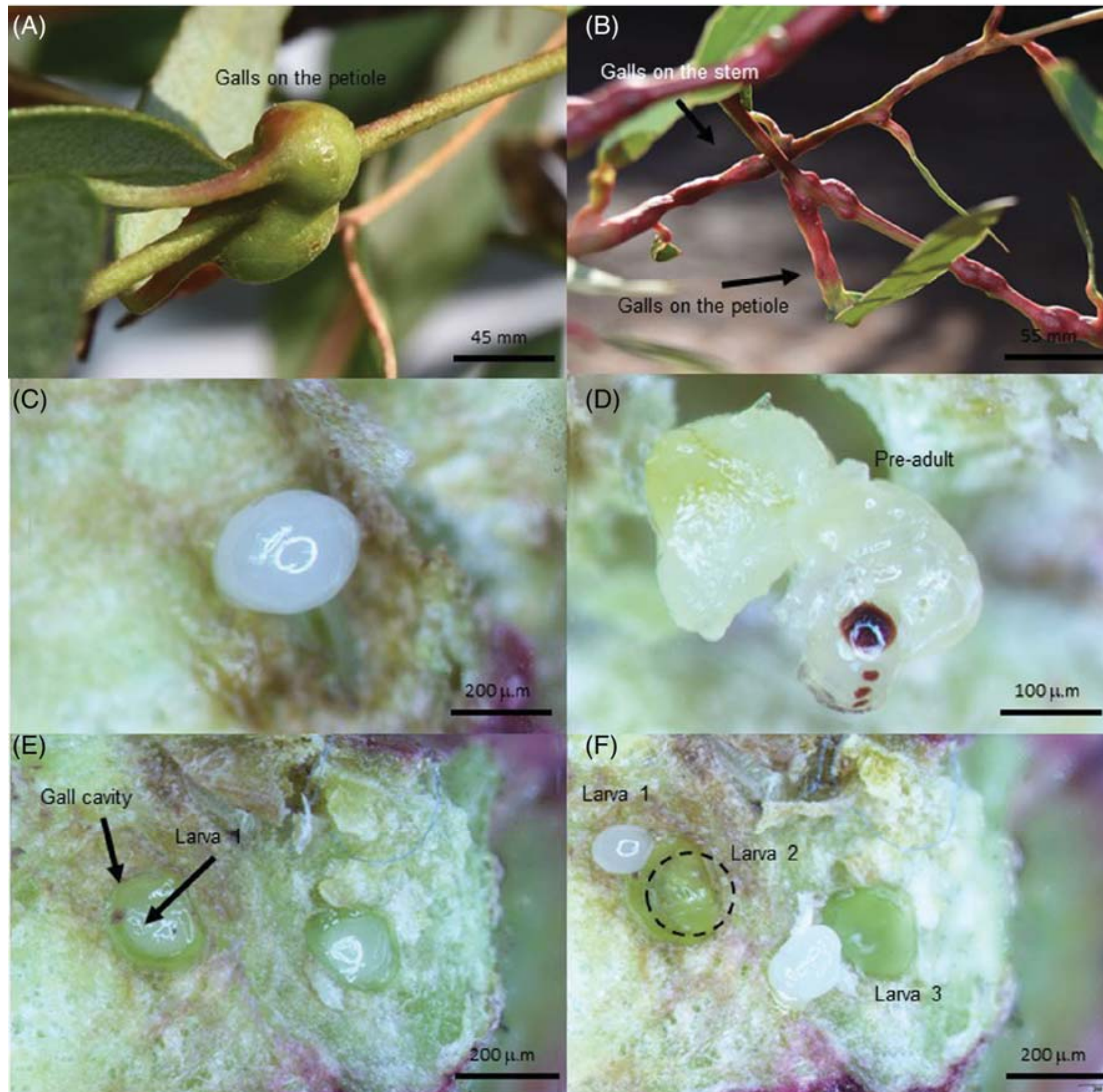

Figure 1. (A) *Leptocybe invasa* gall at the base of the petiole of a *Eucalyptus* leaf. (B) A branch of *Eucalyptus* where the stems and petioles are covered in *L. invasa* galls. This is considered a high level of infestation. (C) A minute indistinguishable larva pulled out of a gall cavity. (D) A pre-adult wasp. (E) Two larvae in the gall cavity. (F) A larva pulled out of the gall cavity; the circle indicates a second larva within the cavity.

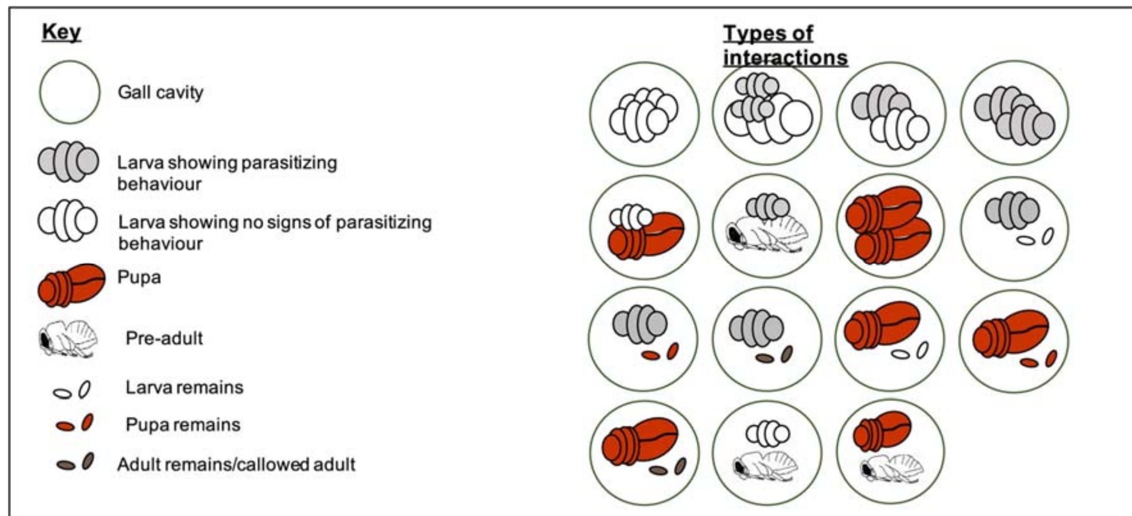

Figure 2. A schematic diagram of the potential types of interactions.

DNA extraction and molecular identification

DNA was extracted from the entire specimen (larva, pupa, pre-adults, insect remains) on the same day that the gall was dissected using the ZyGem DNA extraction kit *Prep*GEM™ insect. Each reaction comprised 20 µl PCR grade water, 4 µl 10x Buffer (Black), 1 µl PrepGEM™ as well as the single larval specimen. Specimens were crushed in the PCR tube using a pipette tip prior to incubation. Incubation was conducted at 75°C for 15 minutes and 95°C for 5 minutes using the Bio-Rad iCycler thermocycler.

A 716 bp sequence of the cytochrome b (*Cyt* b) region of the mitochondrion, from positively identified adult hymenopterans (*L. invasa* Lineage A, *L. invasa* Lineage B, *S. neseri*, *Q. mendeli*, *M. zebrinus* and *M. pretorianensis*) was used to design species specific primers. Using AliView 1.17.1 the cytochrome b sequences of the six species were aligned and primers were designed to have a unique polymorphism on the 3' end, at least (Supplementary Material, Table S2). Where possible primers were designed to amplify fragments of different sizes, which could then be distinguished on an agarose gel.

To test the six primer pairs, DNA from previously identified adult specimens of *L. invasa* Lineage A (MH093325) and *L. invasa* Lineage B (MH093111), *S. neseri* (MT991475), *Q. mendeli* (KX757847), *M. zebrinus* (MT991476) and *M. pretorianensis* (MT991477) were used for PCR. For each species, PCR reactions were optimised to reduce non-specific amplification. To determine the specificity of the primers, DNA from each species was used as template for PCRs using all six primer pairs. Each PCR reaction mix was prepared using 10.8 µl PCR grade water,

2.5 µl PCR Buffer (Roche, Roche Diagnostió, Mannheim, Germany), 2.5 µl dNTP's (10 µM of each dNTP) (Roche), 3 µl $MgCl_2$ (Roche), 0.2 µl FastStart *Taq* polymerase, 1 µl of a 10 µM dilution of each primer (Supplementary Material, Table S1) and 4 µl DNA. The PCR amplifications were performed using a Bio-Rad iCycler with the following PCR protocol: an initial denaturation of 7 minutes at 95 °C, 35 cycles of 1 minute at 95 °C, 1 minute at the species-specific annealing temperature (Supplementary Material, Table S1) and 1 minute at 72 °C, followed by a final extension step of 10 minutes at 72 °C. The PCR products were visualised on 2 % agarose gel using a BioRad Gel Doc™ Ez Imager and the software Image Lab v4.0 build 16. These specimens were then sequenced fully to confirm the results. To identify larvae co-occurring in the same gall, DNA extracted from each larva was used for a PCR reaction with each species-specific primer pair (i.e. six PCR reactions). The above-mentioned PCR reaction mix, protocol and visualization method was used.

Restriction enzymes were optimized to distinguish between the five hymenopteran species and primarily to distinguish between the two *Leptocybe* lineages. A 716 bp fragment of the cytochrome b (*Cyt* b) region of mitochondrial DNA from *Leptocybe* Lineage A and *Leptocybe* Lineage B were viewed in CLC Main Workbench 8 (Version 8.0.1). In CLC Main Workbench, under the tab *Sequence settings*, the tab *Restriction sites* was selected and various restriction enzymes were visually compared to determine which ones would cleave the sequences of *Leptocybe* Lineage A and *Leptocybe* Lineage B at different places in the sequence, thus yielding fragments of different sizes for each lineage. The restriction enzyme *AseI* (New England BioLabs) recognises ATTAT sites and cleaves the DNA between the two Thymine bases (Supplementary Material, Table S2).

In order to use the restriction enzymes, a PCR was performed using the primers CP1 (5'-GAT GAT GAT GAA ATT TTG GAT C -3') (Harry *et al*., 1998) and CB2 (5'-ATT ACA CCT CCT AAT TTA TTA GGA AT -3') (Jermiin & Crozier, 1994) to amplify a 716 bp fragment of the cytochrome b (*Cyt* b) region of mitochondrial DNA. Each PCR reaction mix was prepared using 10.8 µl PCR grade water, 2.5 µl PCR Buffer (Roche, Roche Diagnostics, Mannheim, Germany), 2.5 µl dNTP's (10 µM of each dNTP) (Roche), 3 µl $MgCl_2$ (Roche), 0.2 µl FastStart *Taq* polymerase, 1 µl of a 10 µM dilution of each primer (Supplementary Material, Table S2) and 4 µl DNA. The PCR amplifications were performed using a Bio-Rad iCycler with the following PCR protocol: an initial denaturation of 7 minutes at 95 °C, 35 cycles of 1 minute at 95 °C, 1 minute at 49 °C and 1 minute at 72 °C, followed by a final extension step of 10 minutes at 72 °C. The PCR products were visualised on 2 % agarose gel using a BioRad Gel Doc™ Ez Imager and the software Image Lab v4.0 build 16.

The PCR products were purified using the QIAquick® PCR purification kit (QIAGENA) 25 µl reaction was prepared using 18 µl PCR grade water, 0.5 µl *AseI*, 2.5 µl 10x NE Buffer and 4 µl purified DNA product. The reaction was then placed in a Bio-Rad iCycler with an incubation step of 2.5 hours for 37°C, followed by a inactivation step of 20 minutes at 65°C. Products were run on a 2 % agarose gel and visualised using a BioRad Gel Doc™ Ez Imager and the software Image Lab v4.0 build 16.

Testing the potential gall forming ability of Megastigmus pretorianensis

Leptocybe invasa galled material of various ages was collected from the *Eucalyptus* plantation of the National Zoological Gardens of South Africa at Rietondale, Pretoria, South Africa. The galled material was stored at 4°C in 9.5L ADDIS plastic containers, and emerging *M. pretorianensis* adults were collected and placed in glass vials. Between 6 and 10 *M. pretorianensis* adults were placed in a vial, depending on the availability of *M. pretorianensis* at the time and maintaining an equal ratio of males to females. The *M. pretorianensis* adults were allowed to mate for 4 to 5 days. Seven 6-month-old *Eucalyptus grandis x Eucalyptus nitens* hybrid seedlings in healthy condition (highly susceptible to *L. invasa*), were screened for signs of galling, such as oviposition holes and small gall formations. The seedlings where screened using an illuminated loupe. The seedlings were placed into walk-in cages. The seedlings were planted out into #l pots that contained a mixture of compost and river sand. The walk-in cages that housed the seedlings were covered with shade netting and watered once a day with automated sprinklers. Fine meshed caterpillar sleeves were tied around branches of different aged leaves (two sleeves per seedling). A total of 14 replicates. Different aged leaves were considered to account for any potential age preference of *M. pretorianensis*.

The adults were left in the sleeve until they died. The branch was observed using an illuminated loupe over several months to determine if any gall formation (oviposition holes, small gall/bump like protrusions, any change in colouring around the stem, petioles, midribs and leaf surface) occurred as a result of oviposition by *M. pretorianensis*. As a control, *L. invasa* females were exposed to ungalled *E. grandis* x *nitens* hybrid seedlings, following the same protocol as above. A second control was implemented where *M. pretorianensis* was exposed to trees galled by *L. invasa*. These sleeves were checked twice a week for adult emergences of *M. pretorianensis*.

Results

Species-specific primers and restriction enzymes

The species-specific primers that were designed were able to distinguish between *S. neseri*, *Q. mendeli*, *M. zebrinus* and *M. pretorianensis*. Restriction enzymes were able to distinguish between Lineage A, Lineage B, *Q. mendeli*, *M. zebrinus* and *M. pretorianensis*.

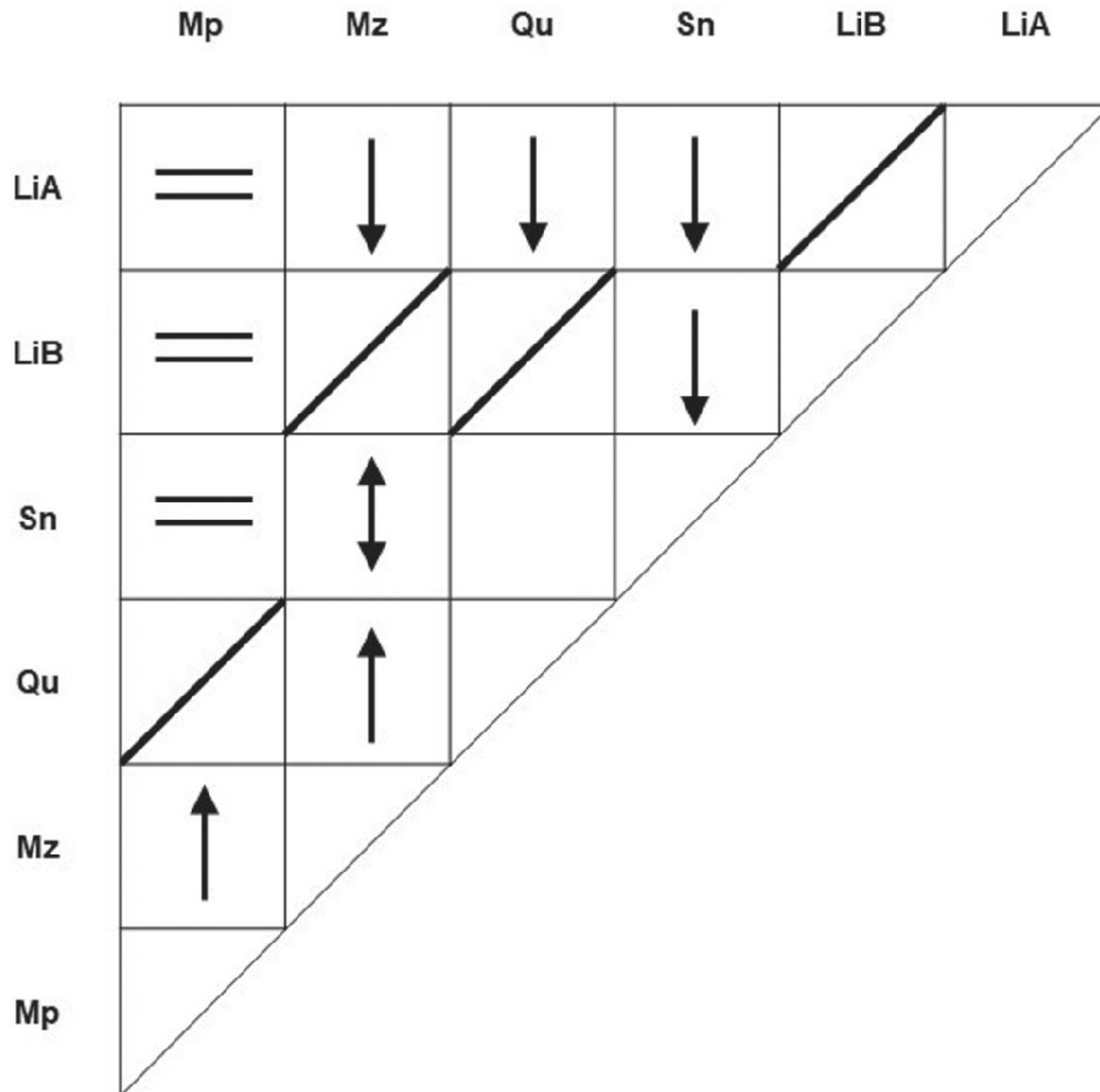

Figure 3. The interactions occurring in the *Leptocybe invasa* gall. The arrow indicates parasitism and the direction of the arrow indicates which species was parasitized. Two horizontal lines indicate that the two larvae occupied a single cavity without confirmation of parasitism. A diagonal line indicates the absence of detected interactions. *L. invasa* Lineage A (LiA), *L. invasa* Lineage B (LiB), *Selitrichodes neseri* (Sn), *Quadrastichus mendeli* (Qm), *Megastigmus zebrinus* (Mz) and *Megastigmus pretorianensis* (Mp).

The roles of Hymenoptera in the Leptocybe invasa complex

Of the 372 galls dissected a total of 156 galls (42%) contained a combination of two or more larvae, pupae, pre-adults or callowed adults. The interacting species were successfully identified in 103 of these galls and the nature of the interactions noted (Figure 3). *Leptocybe* Lineage A and B were never found to co-inhabit a gall, despite being present on the same tree. Both *Leptocybe* lineages were parasitized by the biological control agent *S. neseri*. *Selitrchodes neseri* was also confirmed to parasitize *M. zebrinus*. *Quadrastichus mendeli* was confirmed to parasitize *Leptocybe* Lineage A and *M. zebrinus*. *Megastigmus zebrinus* was confirmed to parasitize *Leptocybe* Lineage A, *S. neseri* and *M. pretorianensis*. *Megastigmus pretorianensis* was not confirmed to parasitize any of the other wasp species, but was found co-inhabiting the galls containing both *L. invasa* lineages and *S. neseri*. In these instances, dissections did not indicate one larva feeding on another but rather two larvae sharing the gall, and the application of species-specific primers did not show one species present in the gut of another (i.e. parasitism).

The gall-forming potential of Megastigmus pretorianensis

Megastigmus pretorianensis did not show oviposition behavior and no plant material developed galls. There were no oviposition marks or evidence of gall formation. *Megastigmus pretorianensis* only emerged from the plant material with *L. invasa* galls.

Discussion

Hymenopteran gall formers can form complex communities within the same gall. In this study, we investigated the community associated with *L. invasa* in South Africa. This community has become more complex since the discovery of a genetically distinct lineage, *L. invasa* Lineage B, together with the already known lineage, *L. invasa* Lineage A (Dittrich-Schröder *et al*., 2018). The community also contains two known biological control agents of *L. invasa*, namely *S. neseri* and *Q. mendeli*, and two species of *Megastigmus*. The study not only confirmed the known gall-forming and parasitic roles of the members of this community, but also revealed previously unknown parasitic interactions. The role of *M. pretorianensis*, however, remains to be fully clarified.

The biology of *M. pretorianensis* was not known at the onset of this study. It was demonstrated in this study that *M. pretorianensis* is not able to develop galls on eucalypts and thus is not a potential pest in eucalypt plantations. *Megastigmus pretorianensis* was described by Doganlar (2015) from wasps collected from *Eucalyptus* galls, but

its role in these galls was not known. The life histories of *Megastigmus* species are difficult to determine because they represent a widespread group containing facultative parasitoids, inquilines and gall formers (Grissell, 1999; Protasov *et al*., 2007; Klein *et al*., 2009).

Megastigmus pretorianensis was found co-inhabiting galls containing both *Leptocybe* lineages A and B and *S. neseri* and may be an inquiline, lethal inquiline or a parasitoid. However, our study was unable to distinguish between these possible roles of *M. pretorianensis*. This could be because galls were dissected before the interactions were evident. *Megastigmus* species belong to the subfamily Megastigminae, where one third of the species are parasitoids (Protasov *et al*., 2007; Klein *et al*., 2009). Globally the *Megastigmus* species are the dominant parasitoids found emerging from *L. invasa* galls, and multiple *Megastigmus* species are confirmed as parasitoids of *L. invasa* (Protasov *et al*., 2007; Zheng *et al*., 2014; Le *et al.* 2020), such as *M. dharwadicus* and *M. vigginali* (Huang *et al*., 2018). This suggests the potential of *M. pretorianensis* as a parasitoid of *L. invasa* in South Africa, as has been assumed before (Huang *et al*., 2018). If *M. pretorianensis* is a parasitoid or a lethal inquiline, it would be of importance to the management of *L. invasa* and would be worth investigating further. Further investigations would include a combination of host specificity tests and oviposition behavioral tests to confirm the role of *M. pretorianensis*.

Selitrichodes neseri, Q. mendeli and *M. zebrinus* were confirmed to parasitize *L. invasa* Lineage A. It was expected that *S. neseri* parasitizes Lineage A, as *S. neseri* was released and established in South Africa as a biological control agent against *L. invasa* Lineage A, before Lineage B was reported present in the country. Before its release in South Africa, studies in quarantine using South African *L. invasa* populations indicated that *S. neseri* could reach parasitism levels of up to 70% (Dittrich-Schröder *et al*., 2014). It was also expected that *Q. mendeli* would parasitize *L. invasa* Lineage A, as it was released as a biological control agent and successfully established in Israel (Kim *et al*., 2008; Bush *et al*., 2017), which is only known to contain *L. invasa* Lineage A (Dittrich-Schröder *et al*., 2018). The confirmation that *M. zebrinus* parasitizes *L. invasa* Lineage A indicates that it may also contribute in the management of this pest. *Megastigmus zebrinus* is also present in Argentina and in Thailand (Huang et al. 2018). Argentina is currently only known to contain Lineage A while both *L. invasa* Lineage A and B are present in Thailand (Dittrich-Schröder *et al*., 2018). Further studies are needed to investigate the prevalence and comparative importance of these three parasitoids of *L. invasa* Lineage A in South Africa.

Only *S. neseri* was confirmed to parasitize *L. invasa* Lineage B. The distribution of *L. invasa* Lineage B, however, does not overlap with all the other parasitoids. *Quadrastichus mendeli* was accidentally introduced into South

Africa and reported in 2017 (Bush *et al*., 2017). Its distribution was limited to the Gauteng province at the time of this study, which did not contain *L. invasa* Lineage B. *Quadrastichus mendeli* co-occurs with both *L. invasa* lineages in Laos (Dittrich-Schröder *et al*., 2018), which offers another opportunity to study the interactions. Possible host association and preference of the different parasitoids on the different *L. invasa* lineages needs to be tested. This would require establishing separate populations of the *L. invasa* lineages in a contained rearing facility or waiting for further natural distribution of the different wasps to co-occur with *L. invasa* Lineage A and B.

Megastigmus zebrinus was found to parasitise *S. neseri*, which highlights its potential as a hyperparasitoid. Hyperparasitism is a competition strategy in parasitoid assemblages (Brodeur *et al*., 2000), when a secondary parasitoid develops on the primary parasitoid (Bruzzone *et al*., 2018). The host range of hyperparasitoids tends to be broader at the species level than that of primary parasitoids (Sullivan, 2009). *Megastigmus zebrinus*, although originally described as a gall former, was later confirmed as a parasitoid within the *Quadrastichodella nova* gall community (Klein, 2009). Its host, however, was not confirmed and it may have parasitized the parasitoids and not the gall former.

We found several direct interactions between the parasitoids in *L. invasa* galls, as well as with *M. pretorianensis*. These included *S. neseri* parasitizing *M. zebrinus*, *M. zebrinus* parasitising *S. neseri* and *M. pretorianensis*, and *Q. mendeli* parasitising *M. zebrinus*. Parasitoid females can usually discriminate between parasitized and non-parasitized hosts and oviposit on non-parasitised hosts (ven Lenteren, 1976; Wylie, 1965; Bruzzone *et al*., 2018). The female may oviposit in a parasitised host due to an excess of parasitoids or a reduction in the host population, which would limit the female options and increase the chances of direct interactions (Bruzzone *et al*., 2018). This results in multiparasitism of the host and the occurrence of interspecific intrinsic competition (Cusumano *et al*., 2016). Intrinsic competition occurs during the larval development (adult-larva or larva-larva) (Harvey *et al*., 2013). Physical interactions and physiological suppression may occur between the competing larvae (Fisher, 1961; Vinson & Hegazi, 1998; Harvey *et al*., 2013). Physical interactions will often occur when a species is required to consume most or all of its host in order to pupate, such as most of the endoparasitoids in the superfamily Ichneumonoidea (Harvey *et al*., 2013).

Parasitoids are often assumed specialists with a narrow host range (Harvey *et al*., 2013). Yet, there are parasitoids that are generalists with a larger host range. Examples of these generalists would include *Aphidius ervi* Haliday (Hymenoptera: Braconidae) and *Praon volucre* Haliday (Hymenoptera: Bracobidae), two generalist parasitoids on aphids (Stilmant *et al*., 2008). The procedures for predicting the host range of herbivorous insects are well

established, but the host range testing procedures for parasitoids are limited (Sands & Van Driesche, 2000; Murray *et al*., 2010; Bush *et al*., 2017). Different methods have been used to assess parasitoid host ranges and the possibility of non-target effects (Van Driesche & Murray, 2004; Murray *et al*., 2010; Bush *et al*., 2017). The relative predictive accuracy of these methods have, however, rarely been assessed (Murray *et al*., 2010). Many studies of gall wasp communities merely list the parasitoids that emerge as parasitoids of the gall former. Our results support the need for caution in assigning ecological roles merely through observing its presence in a gall system.

The study of interactions and roles in gall communities is challenging. The interactions all occur within the confines of the gall. The only way to access the interacting larvae is through dissections. The larvae are minute and morphologically similar, thus making it difficult to differentiate between the species through morphological means. In our study, the development of species-specific primers provided an efficient and cost-effective approach to understand the interactions occurring within the gall community of *L. invasa*. However, species-specific primers are not always able to distinguish between cryptic species or sub-species lineages, such as *L. invasa* Lineage A and B. If this is the case other methods such as restriction enzymes may be more suitable. This study demonstrated the value of such a combination of molecular tools to gain insight into gall biology.

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