Evidence that *Quadrastichodella nova* (Hymenoptera: Eulophidae) is the only gall inducer among four hymenopteran species associated with seed capsules of *Eucalyptus camaldulensis* (Myrtaceae) in South Africa

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Three chalcidoid wasp species, *Megastigmus zebrinus* Grissell (Torymidae), *Quadrastichodella nova* Girault (Eulophidae) and *Leprosa milga* Kim & La Salle (Eulophidae), have each been described independently as gall inducers associated with *Eucalyptus* species (Myrtaceae). The finding that at times they emerge together from seed capsules of river red gum (*Eucalyptus camaldulensis* Dehnhardt) collected at the same site in South Africa, cast doubt on the accuracy of these earlier interpretations. The current study examined the gall inducing abilities of each of the three wasp species. During geographical surveys, all three species coexisted in seed capsules at 16 of the 61 sites sampled. A study of the seasonal emergence pattern of the three species, together with a fourth, locally abundant gall associate, *Aprostocetus* sp., showed that *Q. nova* and *L. milga* emerge during early summer, while the remaining two species emerge in smaller numbers throughout the year. Oviposition trials on sleeved branches of *E. camaldulensis*, from which all insects had previously been excluded, verified that *Q. nova* had the ability to induce galls, while both *M. zebrinus* and *L. milga* failed to do so. Only one type of gall of characteristic structure was encountered, which repudiates the possibility of a second gall inducer, and no indication of inquilinism was found. *Megastigmus zebrinus*, *L. milga* and *Aprostocetus* sp. are thus more likely to be parasitoids. DNA sequences were obtained for the adults of all four these species. By matching the DNA of identified adults with that of juvenile hymenopterans in the galls, it was confirmed that all four hymenopterans species developed within the seed-capsule galls of *E. camaldulensis.* Regrettably, this technique failed to give a clear indication of the exact host relationships between the various gall inhabitants. By dissecting seed capsules at different stages of gall development, the origin of the gall was proven to be in the placenta of one of the locules of a flower bud, and not in a seed or ovule, as previously reported.

Key words: biological roles, oviposition, geographical distribution, seasonal emergence pattern, DNA sequencing.

INTRODUCTION

An Australian tree species, river red gum (*Eucalyptus camaldulensis* Dehnhardt) (Myrtaceae) is valued in South Africa as a general-purpose utility and ornamental tree (Poynton 1979) which is commonly used for fibre production as a hybrid with *E. grandis* (Denison & Kietzke 1993; Van Wyk 1993). It also serves as crucially important source of nectar and pollen to sustain honeybee populations, which are essential for pollinating economically important fruit crops, mainly in the Western Cape Province (Johannsmeier 1993; Allsopp & Cherry 2004). On the other hand, river red gums become invasive in South Africa, especially near water (Forsyth *et al*. 2004; Henderson 2006), the major concern being the large amounts of water they reputedly transpire when growing along watercourses (Henderson 2002; Cambray 2006). Therefore a 'conflict of interests' exists between government agencies that target river red gum for control actions and farmers, beekeepers and foresters who use it.

While these conflicts of interest situations impose limitations on the use of biological control, there are several examples in South Africa where host-

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specific insects that reduce the number of viable seeds have been used successfully for biological control to decrease the invasiveness of trees that are both useful and invasive (Hoffmann *et al*. 2011; Impson *et al*. 2011; Zachariades *et al*. 2011). As a result, an investigation was initiated to identify suitable natural enemies that reduce the number of viable seeds produced by *E. camaldulensis.*

It is standard procedure in the biological control of weeds to undertake a survey of phytophagous organisms associated with the target weed in its introduced, invasive range, before considering the importation of potential biocontrol candidates from the native range of the plant. During surveys of gum species in South Africa in 2000–2001, various unidentified hymenopterans, including a *Megastigmus* sp., were reared from the seed capsules of *E. camaldulensis* and the closely related *E. tereticornis* Smith in South Africa, without having been introduced intentionally (R. Adair, then ARC-PPRI, and L.G. Madire, ARC-PPRI, pers. comm. 2004). At approximately the same time as the local survey, Grissell (2006) reported the discovery of a *Megastigmus* sp. emerging from tiny, seed-like galls in the seed capsules of *E. camaldulensis* in the Western Cape Province of South Africa during 1998. Grissell (2006) believed that *Megastigmus zebrinus* Grissell (Hymenoptera: Torymidae), which he described as a new species of Australian origin, was the inducer of these galls. This made *M. zebrinus* the first recorded gall inducer in the genus (Grissell, 2006). Until then, only seed feeders, parasitoids and partial phytophages, which require a gall former as host but also feed on gall tissue (Murakami 1981; Grissell 1999), had been described in the genus *Megastigmus*. Doğanlar & Hassan (2010) listed 34 Australian *Megastigmus* species that were associated with *Eucalyptus* species, the majority of which had been reared from galls. Many Australian *Megastigmus* species have been reared from galls on several other plant species, although biological information for the genus is very scant (Grissell 1976, 1999, 2006). Remarkably, during 1998, *M. zebrinus* was also reared from the fleshy drupes of cultivated specimens of *Syzygium cordatum* Hochstetter ex Krauss (Myrtaceae), in or near Cape Town (Western Cape Province, South Africa) by J.J. Cillie (The Chalcidoidea Specimen Database of the Biosystematics Division, ARC-Plant Protection Research Institute, Pretoria) and by S. van Noort (Grissell 2006). The morphology of *M. zebrinus* specimens reared from *S. cordatum* differed slightly from those reared from *E. camaldulensis*, but molecular data suggested that they were one species (Grissell 2006).

Further surveys by ARC-PPRI revealed that a second chalcidoid species, *Quadrastichodella nova* Girault (Hymenoptera: Eulophidae), also emerged from the seed capsules of *E. camaldulensis* in various parts of South Africa (The Chalcidoidea Specimen Database of the Biosystematics Division, ARC-Plant Protection Research Institute, Pretoria). In the literature this species (also known as *Flockiella eucalypti* Timberlake) was reported as a gall inducer in the seed capsules of *E. tereticornis* (misidentified as *E. umbellata* (Gaertn.) Domin.) in California, U.S.A. (Flock 1957; Timberlake 1957). Flock's (1957) description of the gall is very similar to the gall attributed to *M. zebrinus* (Grissell 2006), in that it develops internally, is not visible from the outside, and roughly resembles the seeds of the host plant. Most members of the phytophagous genus *Quadrastichodella* Girault (1913) are associated with *Eucalyptus* species (La Salle 1994a; Kim *et al.* 2005; Kim & La Salle 2008). *Quadrastichodella nova* was originally described from Australia (Girault 1922) and in addition to the U.S.A. and South Africa, has since also been recorded from Argentina, Spain and Israel (Bouček 1977), Turkey (Doğanlar & Doğanlar 2008) and the Republic of the Congo (The Chalcidoidea Specimen Database, ARC-PPRI). Doğanlar & Doğanlar (2008) were first to report galling by *Q. nova* in *E. camaldulensis*, notably in Turkey, although unpublished label data for *Q. nova* from *E. camaldulensis* had existed in South Africa 10 years earlier (HYMC023, South Africa, Grootkloof, SW of Buffelspoort Dam, 25.51S 27.24E, 22/02/1998, S. Neser, ex fruits of *Eucalyptus camaldulensis*) (The Chalcidoidea Specimen Database of the Biosystematics Division, ARC-Plant Protection Research Institute, Pretoria).

While attempting to resolve the question whether *M. zebrinus* and *Q. nova* each independently produces its own gall in the seed capsules of *E. camaldulensis* in South Africa, or whether one of the two chalcicoid species was in reality an inquiline or a parasitoid, two more chalcidoid species were found emerging from seed-capsule galls in *E. camaldulensis* in South Africa in relatively large numbers. These were also included in the investigation. One of the two new species was subsequently described as a species in a new genus: *Leprosa milga* (Kim & La Salle) (Eulophidae:

Tetrastichinae), the type specimen of which originated from Stellenbosch, Western Cape, South Africa (Kim & La Salle 2008). The earliest known specimens were, however, reared by S. Neser from seed capsules of *E. camaldulensis* collected at Rietondale Research Centre in Pretoria, Gauteng during August 2002, and from *E. camaldulensis* seed capsules collected by A.B.R. Witt from Colesberg and Hanover, Northern Cape Province, during November 2003 (The Chalcidoidea Specimen Database of the Biosystematics Division, ARC-Plant Protection Research Institute, Pretoria). *Leprosa milga* was regarded as a native of Australia and, based on the available host records at the time of its description, it was regarded as a gall inducer in the seed capsules of *E. camaldulensis* (Kim & La Salle 2008), thus bringing the number of reputed gall inducers in the seed capsules of *E. camaldulensis* in South Africa to three. The fourth species to emerge from seed-capsule galls of *E. camaldulensis* as part of this investigation was an unidentified species of *Aprostocetus* (Eulophidae: Tetrastichinae) (det. O.C. Neser).

We report here on the results of the investigation into the life history of the assemblage of chalcidoids associated with internal galls in the seed capsules of *E. camaldulensis* in South Africa and, in particular, on the question which of the species were gall inducers. Any implications of the findings for the potential biological control of *E. camaldulensis* will be published separately.

MATERIAL AND METHODS

Geographical distribution of chalcidoid species in South Africa

To get an indication of how widely the four hymenopteran species were distributed in South Africa, and in which areas they co-occurred, surveys of *Eucalyptus camaldulensis* were undertaken in the Gauteng, Mpumalanga, North West, Limpopo, Free State and Northern and Western Cape Provinces of South Africa between 2003 and 2008. Although *E. camaldulensis* also occurs in KwaZulu-Natal and the Eastern Cape Province, logistics prevented surveys in these two provinces. Wherever possible, localities were revisited repeatedly, to ensure that insects emerging during different seasons were detected.

Natural hybridization between *E. camaldulensis* and the closely related *E. tereticornis* (forest red gum), which also occurs in South Africa (Poynton 1979), gives rise to offspring that have characteristics intermediate between the two species (Brink 2008). Although during this study only trees that had more characteristics of *E. camaldulensis* than of *E. tereticornis* were surveyed for seed-capsule galls and their inhabitants in South Africa, the possibility of including some hybrids in the surveys cannot be discounted. It would therefore be more accurate to refer to 'the *E. camaldulensis* complex' where geographical surveys in South Africa are concerned but, for the sake of briefness, the name *E. camaldulensis* is used instead throughout the paper.

When sampling, approximately 250 ml to 500 ml of seed capsules from one tree per locality were kept in individual carton emergence boxes in an office at the Rietondale Weeds Laboratory, Pretoria, under ambient temperatures and light conditions. The room was not humidified and the capsules in the emergence containers gradually dried out. Insects that emerged from each sample were recorded. The results (Table 1) were analysed to indicate the location, number and date of sample collections, and the chalcidoid species that emerged per site. Other hymenopterans that emerged only rarely are listed in Table 2. Voucher specimens of all species that emerged during this study were deposited in the National Collection of Insects, Biosystematics Division, ARC-Plant Protection Research Institute, Pretoria.

Evaluation of the gall-inducing ability of the various gall associates

The ability of an insect species to induce galls in a particular plant species can be verified by observing oviposition and the development of galls in the plant when the appropriate part of the plant, from which all other arthropods have been excluded, is presented to sexually mature adults. In contrast to gall inducers, the females of inquilines will only oviposit in a gall that has already been produced by another species (Brooks & Shorthouse 1998), while parasitoids would require a host insect.

The hymenopterans under investigation emerged only from galls in fully developed seed capsules of *E. camaldulensis*. As gums normally produce seeds only after they have reached several years of age (Hodgson 1976; Chambers *et al.* 1997; Jordan *et al.* 1999), laboratory rearing of the insects and experimentation with individual species was deemed impractical. Consequently this study was largely

Table 1. Emergence of hymenopterans from samples of seed capsules of the *Eucalyptus camaldulensis* complex collected at different localities in South Africa (x indicates emergence, with no reference to numbers). *Mz = Megastigmus zebrinus*; *Qn = Quadrastichodella nova*; *Lm = Leprosa milga*; *Ap = Aprostocetus* sp.

Province	Locality	Sample date(s)	No. samples	Wasp species			
				Mz	*Qn*	*Lm*	*Ap*
Gauteng	Rietondale, Pretoria 25°43′51″S 28°14′31″E	Apr 07–Mar 08	26	x	x	x	x
	Univ. Pretoria 25°45′11″S 28°15′11″E	Apr 07–Mar 08	26	x	x	x	x
	Rooihuiskraal, Pretoria 25°52′41″S 28° 8′25″E	Apr 07–Mar 08	26	x	x	x	x
	Lynnwood Ridge, Pretoria 25.46S 28.17E	Dec 03	1	x			
	Bronkhorstspruit 25°53′37″S 28°40′59″E	Jan 06	1				
Limpopo	Modimolle/Nylstroom 24°40′23″S 28°30′15″E	Nov 04; Oct 05; Mar 06	3	x		x	
	Bela Bela/Warmbaths 24°53′03″S 28°18′02″E	Jan 05	1				
	Potgietersrus 24°09′18″S 29°06′57″E	Jan 05	1				
	Bolobedu 23°37′23″S 30°20′44″E	Jan 05	1				
	Klein Kariba 24°50′55″S 28°19′40″E	Nov 04	1				
Mpumalanga	Waterval-Onder 25°26′51″S 30°57′59″E	Jan 05	1	x			
	Long Tom Pass 25°06′25″S 30°29′40″E	Jan 05	1				
	Ohrigstad/Lydenburg rd 24°51′22″S 30°34′24″E	Jan 05	1				
	Lydenburg 25°06′49″S 30°28′16″E	Dec 04	1			x	
	Machadodorp 25°36′51″S 30°17′05″E	Jan 05	1				
North West	Grootkloof, Buffelspoort 25.51S 27.24E	Feb 1998	1	x	x		
	Brits 25.39S 27.46E	Aug 03	1		x		
	Sparkling Waters 25°49′53″S 27°24′41″E	Nov 04	2		x		
	Seremodi	Sep 08	1		x		
	Boekenhoutkloof 25.24S 28.17E	Nov 03	1		x		
	Bloemhof 27°39′06″S 25°35′43″E	Dec 04	1	x			
	Klerksdorp 26°52′04″S 26°37′52″E	Dec 04	1	x	x		
Free State	Kroonstad 27°46′03″S 27°13′29″E	Dec 04; Feb 07	2				
	Edenburg 29°44′2″S 25°57′17″E	Aug 07	1	x	x	x	
	Reitz 28°0′51″S 28°22′23″E	Dec 05	1				
	Sasolburg 27°08′15″S 27°31′16″E	Dec 04	1	x	x		
	Koppies 27°12′52″S 27°31′14″E	Dec 04	1	x			
	Winburg 28°30′41″S 27°00′38″E	Dec 04	1	x	x		
	Bloemfontein 29°03′06″S 26°13′35″E	Dec 04	1	x			
	Springfontein 30°15′38″S 25°42′33″E	Dec 04	1	x			
Northern Cape	Colesberg 30°42′25″S 25°06′34″E	Nov 03; Dec 04; Feb 07; Oct 07	4	x	x	x	
	Colesberg 26 km S 30°52′49″S 24°50′46″E	Nov 03; Dec 04; Feb 07; Oct 07	4	x	x	x	
	Hanover 25 km S of 31°13′32″S 24°15′16″E	Nov 03; Dec 04	2	x	x	x	
	Matjiesfontein 33°13′47″S 20°34′47″E	Nov 03	1	x			
	Richmond 31°24′46″S 23°56′08″E	Dec 04	1		x		
	Three Sisters 31°53′20″S 23°04′26″E	Dec 04	1		x		
	Three Sisters/Victoria West 31°40′28″S 23°05′02″E	Dec 04	1		x		
	Britstown 30°35′19″S 23°30′07″E	Dec 04	1				

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Table 1 (continued)

Province	Locality	Sample date(s)	No. samples	Wasp species			
				Mz	*Qn*	*Lm*	*Ap*
	Strydenburg 29°57′12″S 23°40′13″E	Dec 04	1				
	Hopetown 29°37′32″S 24°04′47″E	Dec 04	1	x	x		
Northern Cape	Springbokkamp 29°05′03″S 24°36′24″E	Dec 04	1	x	x		
	Warrenton 28°05′50″S 24°52′06″E	Dec 04	1	x			
	Raap en Skraap 28°37′38"S 19°30′22″E	Apr 08	1		x		
Western Cape	Welverdiend 32°04′52″S 18°49′38″E	Dec 05; Jan 07; Oct 07	3	x	x	x	
	Algeria road 32°23′17″S 18°56′34″E	Dec 05	1	x			
	Trawal 31°55′27″S 18°40′56″E;	Dec 05	1	x			
	Klawer 31°47′03″S 18°37′02″E	Dec 05	1				
	Citrusdal 15 km S 32°25′22″S 18°57′34″E	Dec 05; Jan 07; Oct 07	3	x	x	x	
	Citrusdal 5 km S 32°32′44″S 19°00′32″E	Dec 05; Jan 07; Oct 07	3	x	x	x	
	Citrusdal Caravan Park 32º35′33″S 19º00′39″E	Oct 07	1	x	x	x	x
	Tulbagh /Worcester 33°29′15″S 19°11′48″E	Dec 05	1				
	Wolseley /Worcester 33°37′16″S 19°22′32″E	Dec 05	1			x	
	Pampoenstalletjie (Worcester/Robertson) 33°40′14″S 19°33′29″E	Dec 05; Jan 07	2	x	x	x	
	Bonnievale 33°56′40″S 20°04′48″E	Dec 05; Jan 07	2	x	x	x	x
	Sonderend River 34°04′51″S 20°05′38″E	Dec 05; Jan 07	2			x	
	Bredasdorp/Waenhuiskrans 34°34′56″S 20°06′54″E	Dec 05	1	x			
	Wolfdrif 32°03′01″S 19°04′00″E	Jan 07; Oct 07	2	x	x	x	x
	Lorraine 32°03′01″S 19°03′07″E	Jan 07; Oct 07	2		x		
	Travellers Rest 32°04′15″S 19°04′05″E	Jan 07; Oct 07	2	x	x		
	Somerset West 34.05S 18.51E	Mar 1998	1	x			
	Rhenish School, Stellenbosch 33°56′45″S 18° 51′21″E	Dec 04; Nov 05; Jan 06; Mar 06; Feb 07; Sep 08	6	x	x	x	
Total	**61**			**35**	**31**	**19**	**6**

limited to field observations, field trials and microscopic examination of dissected flower buds, flowers and seed capsules.

Emergence pattern of the gall inhabitants

All adults required for oviposition trials to evaluate the gall-inducing ability of the various alleged gall inducers, had to be reared from field-collected material. Therefore information about the period and pattern of emergence in each chalcidoid species was required, which in turn necessitated regular sampling of emergences from seed capsules of the host plant throughout the year.

To this end, regular fortnightly samples were taken from three selected *E. camaldulensis* trees in Pretoria, from April 2007 until March 2008, in areas where all four gall-associates had been recorded previously. These trees were located at Rietondale (25 °43′50.97″S 28 °14′30.64″E), University of Pretoria experiment farm (25 °45′11.09″S 28 °15′11.02″E) and a security complex, Craddock Park, in Rooihuiskraal North (25 °52′41.39″S 28 ° 8′25.20″E). Each sample consisted of approximately 250–500 ml of fully developed seed capsules per test tree, and emergence boxes were used to collect any insects that emerged from the material until December 2008. The number of seed capsules per sample was recorded. Insect emergences were recorded per locality per month, and plotted in two ways: (a) showing the total number of individ-

Table 2. Incidentally collected hymenopteran species from seed capsules of *Eucalyptus camaldulensis*

Accession number	Collection locality	Species (family)
AcSN2914	Citrusdal 15 km S, 32°25′22″S 18°57′34″E	*Oomyzus* sp. (Eulophidae: Tetrastichinae)
AcSN2915 AcSN2916	Citrusdal 15 km S, 32°25′22″S 18°57′34″E	*Eupelmus* spp. (Eupelmidae)
AcSN2917	Citrusdal 15 km S, 32°25′22″S 18°57′34″E	*Aphelinus* (Aphelinidae)
AcSN2918 AcSN2919 AcSN2943 AcSN2947	Wolfdrif, 32°03′01″S 19°04′00″E	*Eupelmus* spp. (Eupelmidae)
AcSN2920	Wolfdrif, 32° 03′ 01″S 19°04′00″E	*Eupelmus* sp. (Eupelmidae) *Oomyzus* sp. (Eulophidae: Tetrastichinae) Gen. sp. unknown (Eulophidae: Tetrastichinae)
AcSN2922	Pampoenstalletjie (Worcester/ Robertson), 33°40′14″S 19°33′29″E	Gen. sp. unknown (Pteromalidae) *Pachyneuron* sp. (Pteromalidae)
AcSN2933	Welverdiend, 32°04′52″S 18°49′38″E	*Gryon* sp. (Scelionidae)
AcSN2927	Rietondale Pretoria, 25°43′39″S 28°14′13″E	Scelionidae
AcSN2927	Rietondale Pretoria, 25°43′39″S 28°14′13″E	Scelionidae
AcSN2944	University of Pretoria exp. Farm, 25°45′11.09″S 28°15′11.02″E	Scelionidae
AcSN2937	Rhenish School, Stellenbosch, 33°56′45″S 18°51′21″E	*Enoggera* sp.
AcSN2933	Bonnievale, 33°56′40″S 20°04′48″E	*Mesopolobus* sp. (Pteromalidae)
AcSN2936	Pampoenstalletjie (Worcester/ Robertson), 33°40′14″S 19°33′29″E	*Mesopolobus* sp. (Pteromalidae)

uals of each chalcidoid species that emerged each month, by combining the three sample sites (Fig. 1A, B, C, D), and (b) showing the total number of individuals of each chalcidoid species that emerged at each of the three sample sites, by combining the samples for the entire monitoring period (Fig. 2). The number of hymenopterans was expressed as individuals per 100 seed capsules, and a base-10 logarithmic scale was used to accommodate the large range in emergence numbers.

Laboratory observation of oviposition behaviour of adults

In preparation for the oviposition trials, it was also necessary to ascertain which developmental stages of the eucalypt reproductive structures were acceptable for oviposition by each of the chalcidoids. For this purpose, the behaviour of adults of *M. zebrinus*, *Q. nova*, *L. milga* and *Aprostocetus* sp. was observed under a Leica MZ8 stereo microscope when they were presented with segments of eucalyptus shoots containing flower buds, flowers and seed capsules in glass vials. *Megastigmus zebrinus* was the only species in which females, as well as males, were observed; the four eulophid species were regarded as uniparental, with no male specimens in collections in South Africa or referred to in descriptions (Timberlake 1957; Kim & La Salle 2008).

A number of flower buds, into which females of any species had been seen to oviposit, were dissected to observe the morphology of the egg and the position in the flowerbud in which it had been deposited. Such dissections were made at different intervals after oviposition, but the abscised flower buds shrivelled after about three days and were then no longer suitable for observations.

Oviposition trials in sleeve cages on living trees

To verify which of the chalcidoid species were able to induce galls in the absence of other insect

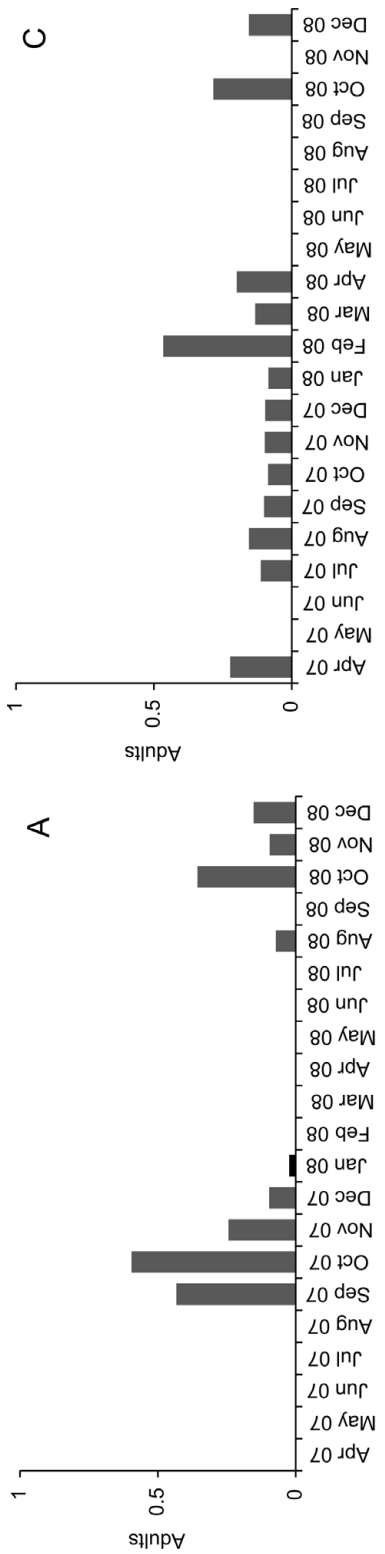

Fig. 1. Emergence patterns of wasps (adults per 100 capsules) from seed capsule samples collected from three *Eucalyptus camaldulensis* trees near Pretoria. **A** = *Quadrastichodella nova*; **B** = *Leprosa milga*; **C** = *Megastigmus zebrinus*; **D** = *Aprostocetus* sp.

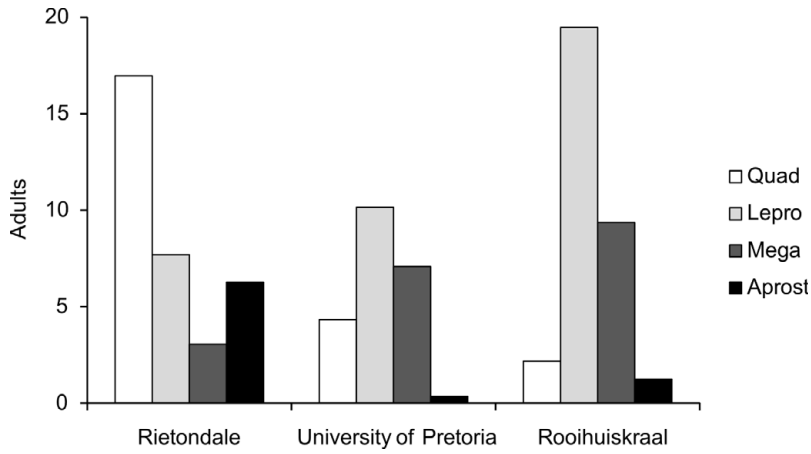

Fig. 2. Cumulative numbers of adults that emerged per 100 capsules for each of the four chalcid species at each of the three samples sites over the entire monitoring period (April 2007–December 2008). Quad = *Quadrastichodella nova*, Lepro = *Leprosa milga*, Mega = *Megastigmus zebrinus*, Aprost = *Aprostocetus* sp.

species, oviposition trials were carried out in gauze sleeves on intact branches on *E. camaldulensis* trees.

This trial was first run during the summer of 2006/2007 (before the seasonal emergence pattern of the various chalcidoids had been studied), using two of the test trees mentioned above, one at Rietondale and the other at the University of Pretoria. On each of the two test trees, the distal sections of 22 branches bearing the youngest discernible flower buds were covered with nylon gauze bags (sleeves), which were tied with string at both ends to enclose the flower buds. Any insects present at that time were squashed or removed manually from the sleeved branches. This excluded all insects while the flower buds developed within the sleeves.

When the flower buds in the sleeves had reached a size considered suitable for oviposition (as determined during laboratory observation of the oviposition behaviour of the insects), the sleeves were assigned to four different treatments. One group (two replicates per tree) was assigned as a control, and these sleeves were left without insects. The other three groups were stocked with batches of adults (<24 h old) of either *M. zebrinus*, *Q. nova* or *L. milga*, obtained from field-collected seed capsules. The exact number of replicates for each species eventually depended on availability (Table 3). Ten females (or sometimes only 3–5, when in short supply) of *Q. nova* were placed in each of three sleeves on the Rietondale tree and one sleeve on the University of Pretoria tree, while 10 females of *L. milga* were placed in each of eight sleeves on the Rietondale tree and nine sleeves on the University of Pretoria tree. The sleeves were then tied up. Several more sleeves were originally stocked with insects, but broke off the trees, or the branches died prematurely; these were excluded from the analysis. In the case of *M. zebrinus*, which did not emerge in large numbers at any one time, eight replicates were carried out on the Rietondale tree and four on the University of Pretoria tree, and each sleeve was stocked with roughly equal numbers of males and females. However, the number of available individuals of *M. zebrinus* was sometimes limited to as few as two females per sleeve. *Aprostocetus* sp. was not included in this trial due to the emergence of insufficient numbers of individuals from field-collected seed capsules.

The first trial series was supplemented with additional treatments during the summer of 2011/2012, when the timing could be improved due to a better understanding of the seasonal emergence pattern of the individual chalcidoid species (see above). The methodology was the same as for the first series, with the following exceptions: Sleeves were set up only in the University of Pretoria tree, since the tree at Rietondale had died during 2008. There were eight sleeves with *Q. nova* females and five with *L. milga* females, but *M. zebrinus* was not included during the 2011/2012 trial series because the numbers that emerged were insufficient and the genders were not synchronized. Even though the two trial series were not identical, the results can still be regarded as valid, since galls can be either present or absent,

Table 3. Number of galls initiated in reproductive structures of *Eucalyptus camaldulensis* on intact branches when exposed to adults of different hymenopteran species in gauze sleeves. Replicates in which the branch died or the sleeve disappeared before the contents were examined, were excluded. UP = University of Pretoria, Rd = Rietondale.

Date of trial series		2006/07			2011/12		
Hymenopteran species	Test tree	No. of sleeves (replicates)	Total no. of females	No. of sleeves in which galls were initiated	No. of sleeves (replicates)	Total no. of females	No. of sleeves in which galls were initiated
Quadrastichodella nova	UP	1	11	1	8	80	7
	Rd	3	24	1			
Leprosa milga	UP	9	54	0	5	60	0
	Rd	8	38	0			
Megastigmus zebrinus	UP	4	34	0	0	0	0
	Rd	8	59	0			
No hymenopterans (control)	UP	2	0	0	4	0	0
	Rd	2	0	0			

with no gradations in-between, and no comparison between the two series would therefore be necessary.

During the first trial series, apart from a few flower buds or seed capsules that were removed for microscopic inspection from some of the sleeves at approximately two-month intervals, the sleeves were left in position for almost a year, to allow all hymenopterans to complete their development and emerge from the capsules. The sleeves with their contents were then removed and examined in the laboratory for dead or live hymenopterans, while all flower buds, flowers and seed capsules were dissected and microscopically examined for galls, the remains of juvenile hymenopterans or emergence holes in the capsules.

During the second trial series, representative sleeved branches were also sampled one and two weeks after the hymenopterans had been added. However, all the sleeves were removed within two or three months from the stocking date. This was necessitated by an increase in the mortality of eucalyptus flower buds, flowers and capsules when sleeved, and evidence that sleeved shoots were breaking off due to wind or vandalism. Despite this period being too short for most of the hymenopterans to have emerged from the galls, it was nevertheless regarded as sufficient for the purpose of this investigation, since the objective was to determine whether galls could be induced, and galls were already visible upon dissection two weeks after the insects had been added.

Dissection of flower buds, flowers and seed capsules containing galls

The examination of dissected flower buds, flowers and seed capsules of *E. camaldulensis* was intended to clarify several aspects: (a) whether the structures were galled; (b) the morphology and development of the gall; (c) whether more than one type of gall could be distinguished, which would indicate that more than one hymenopteran species was a gall inducer since gall morphology is determined by the identity of the gall inducer (Dawkins 1982; Dreger-Jauffret & Shorthouse 1992; Stone & Schönrogge 2003); (d) whether an inquiline was present, as indicated by galls within other galls, or by the anatomical modification of the original gall by the inquiline (Brooks & Shorthouse 1998; Ferraz & Monteiro 2003; Medianero *et al.* 2007; van Noort *et al.* 2007), and possibly by the larva of the original gall inducer

being stung to death or being squashed by the proliferation of tissues in the secondary gall (Shorthouse 1973; Brooks & Shorthouse 1998); (e) identification of the juveniles of different hymenopteran species, and their interactions.

Because the galls in the seed capsules of *E. camaldulensis* are not visible externally, direct observations of the development of the gall and its inhabitants are problematic. Therefore, large numbers of reproductive structures had to be collected randomly for examination. Approximately 4000 individual reproductive structures (flower buds, flowers and seed capsules) of *E. camaldulensis*, representing different developmental stages and samples from different geographical regions in South Africa, were dissected between 2006 and 2011. Attention was paid to all the aspects mentioned in the previous paragraph.

DNA sequencing

In order to discriminate between parasitoids and hosts during their juvenile stages (the only time when they can be seen to interact), an attempt was made to identify the individuals involved in interactions by matching their DNA with that of identified adults (Goolsby *et al*. 2001), to assist in the interpretation of the host relationships between the different species.

DNA was extracted using *prep*GEM™ Insect ZyGEM DNA extraction (ZyGEM, Hamilton, New Zealand) following the manufacturers' instructions. Targeting the cytochrome *b* region of the mitochondrial DNA (mtDNA) the primers CP1 (5'-GAT GAT GAA ATT TTG GAT C-3') (Harry *et al.* 1998) and CB2 (5'-ATT ACA CCT CCT AAT TA TTA GGA AT-3') (Jermiin & Crozier 1994) were used to conduct polymerase chain reaction (PCR). A Bio-Rad iCycler was used to conduct the PCR using the following conditions: 95 °C for 7 min, 35 cycles of (95 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min), 72 °C for 10 min and 4 °C hold. PCR reactions were prepared as a total volume of 25 μl using 10 PCR Buffer, 25 mM $MgCl_2$, 10 μM of each dNTP, 30 pmol of each PCR primer, and 1 unit of Roche FastStart Taq DNA Polymerase and 4 μl of genomic DNA/RNA mix. Subsequently PCR products were visualized on a 2 % agarose gel and then purified using the Roche High Pure PCR Product Purification kit (Version January 2008, Cat No. 11732676001). DNA cycle sequencing was performed, using the purified PCR products, under the following conditions: 95 °C for 1 min, 35 cycles of (95 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min) and 4 °C hold, using an ABI PRISM® BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA. U.S.A.). The Applied Biosystems' Ethanol/Sodium Acetate/EDTA precipitation protocol was used to clean the cycle sequencing products prior to analysis using an ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems). Sequenced products were edited, aligned and analysed using the Staden package (Staden 1996), CLUSTALX version 1.81 (Thompson *et al*. 1997) and BIOEDIT version 7.0.1 (Hall 1999), respectively. MEGA version 5.0 (Tamura *et al.* 2011) was used to construct a neighbour joining (NJ) tree (Fig. 3) with 1000 bootstrap replicates and the Kimura 2-parameter substitution model as well as to calculate uncorrected pairwise DNA distances. A sequence of *Ceratosolen arabicus* (Hymenoptera: Agaonidae) downloaded from GenBank (Accession number AJ235231.1) was used as an outgroup.

RESULTS

Geographical distribution of chalcidoid species

Of the 61 sites sampled during the five-year period, seed capsules from 47 localities yielded specimens of one or more of the following four hymenopteran species: *Megastigmus zebrinus*, *Quadrastichodella nova*, *Leprosa milga*, and *Aprostocetus* sp. (Table 1). Seven of the sites yielded all four species and another nine yielded all except for *Aprostocetus* sp. *Megastigmus zebrinus* was the only species that was recorded from all provinces surveyed. *Aprostocetus* sp. was only recorded from Gauteng and Western Cape Provinces, *Q. nova* from all provinces except Limpopo and Mpumalanga and *L. milga* from all except the North West Province. The majority of samples from which none of the four species emerged originated from Limpopo and Mpumalanga Provinces.

Emergence patterns of the various chalcidoid species

Based on adult emergence patterns, two groups of chalcidoid species could be distinguished (Fig. 1). The first group, consisting of *Q. nova* and *L. milga*, had a well-defined emergence period, with a distinct peak during early summer (September to December), with emergence of *L. milga* apparently slightly delayed relative to *Q. nova* (Fig. 1A, B). Further emergence of *L. milga* occurred from February to April although this was

Fig. 3. Neighbour joining tree showing the linking of larval and pupal specimens to positively identified adult hymenopterans. Values above branches indicate uncorrected pairwise DNA distances whereas values below branches indicate bootstrap support (shown only if >75 %).

only noted at two of the three sample localities. Both these species continued to emerge for up to 9 months after the last seed capsules had been collected. At two of the three localities (Rooihuiskraal and University of Pretoria), and overall, the numbers of *L. milga* distinctly exceeded those of *Q. nova*, while the opposite was true for the remaining locality (Rietondale) (Fig. 2).

The second group, represented by *M. zebrinus* and *Aprostocetus* sp., emerged over an extended

period but, apart from this, the two species had little in common (Fig. 1C, D). For *M. zebrinus*, there were emergences during all months except for May and June, with one apparent peak in February, and adults emerged for up to 9 months after the last seed capsules had been collected. While *M. zebrinus* was common at all three localities, *Aprostocetus* sp. was abundant at only one of the three localities, Rietondale (Fig. 2), from where it emerged during all months except for May (Fig. 1D). However, at the remaining localities it emerged in such low numbers that no pattern was apparent. No specimens of this species emerged more than a month after the last sample of seed capsules had been collected.

Oviposition by the various chalcidoid species

The only females that were seen to probe with their ovipositors and probably to oviposit on detached shoots of *E. camaldulensis* in the laboratory were *Q. nova* and *L. milga*. They inserted their ovipositors only into fully developed flower buds and flowers.

The results of the two series of oviposition trials in sleeve cages on living trees are shown in Table 3. During both series, galls were found only in the sleeves stocked with *Q. nova*. Galls in different stages of development were found in the dissected seed capsules in nine out of the 12 sleeves (75 %) stocked with *Q. nova*; in some cases 30 or more galls were produced per sleeve. In contrast, no galls developed, and no oviposition marks were visible, in any of the seed capsules that developed from the flower buds in either the 12 sleeves that were exposed to specimens of *M. zebrinus* or in the 22 sleeves that were exposed to *L. milga*. None of the eight control sleeves, without hymenopterans, yielded any galls, nor were there any indications of development of wasps.

Structure and development of the seed-capsule galls

The dissections confirmed the descriptions by Hodgson (1976) and Poynton (1979), that a healthy flower bud of *E. camaldulensis* contains an ovary comprising between three and five chambers or locules, each of which contains a few ovules in the basal part of the ovary, and a large amount of infertile ovulodes. All of these are attached to a common, central placenta. As a flower bud matures into a flower and this, in turn, produces a seed capsule, the fertilized ovules give rise to seeds (normally about one or two seeds per locule), while the unfertilized ovules together with the ovulodes give rise to chaff (Fig. 4A).

Although it was not possible to observe in sequence the different stages in the development of galls in detached buds and capsules, the development process could be reconstructed by arranging the 'snapshots in time' presented by each of the 4000 or so dissections until they followed logically one upon the other. The early developmental stages of galls could be witnessed during spring to early summer (September–December) in the flower buds and flowers, while the mature galls, containing fully developed larvae, were found during early or late summer (October–March) in the seed capsules of different ages.

Dissections show that the gall originates in the placenta of one of the locules in the seed capsule of a flower bud, where the eggs of *Q. nova*, with their distinctive long filament, are inserted. One or two days after oviposition, the area surrounding the egg becomes swollen and green, and later contains a colourless, spherical larva living in soft, juicy, green tissue without a hard wall. Still later the green gall starts protruding from the placenta, with 1–3 undeveloped ovules or ovulodes attached to its surface (Fig. 4B). These ovules or ovulodes, whose point of attachment to the placenta is incorporated into the developing gall, do not develop any further but remain visible as thin, later brown, scales on the surface of the gall (Fig. 4C).

As the gall grows, it protrudes further from the placenta until its final size is equal to or slightly larger than that of a seed (Fig. 4A, D), reaching almost the entire distance from the placenta laterally to the locule wall, but with room left above and/or, below the gall; this is largely in accordance with Grissell (2006), although he believed the galls to have been induced by *M. zebrinus*. The gall is roughly spherical, but if multiple galls develop in the confined space in the same locule, their shapes can be modified by adjacent galls. A gall might compress some of the chaff particles in the locule or arrest their development, but has no noticeable influence on the size of other seeds in the capsule. The colour of the gall changes from green to off-white to light brown, often partially or fully covered by the papery, dark-brown remnants of ovules on its surface (Fig. 4D). During its development the contents of the gall changes from watery, gelatinous tissue into drier, firm, green nutritive tissue, and a larval chamber develops in its centre.

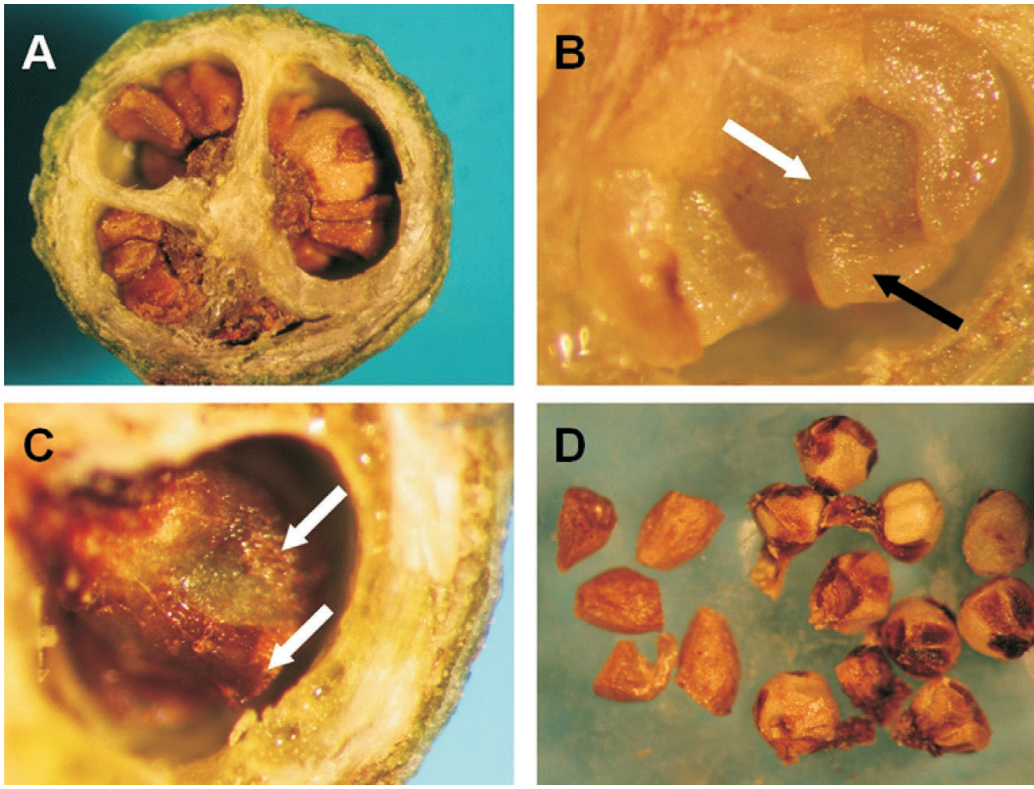

Fig. 4. Galls caused by *Quadrastichodella nova* in the seed capsules of *Eucalyptus camaldulensis*: **A**, transverse section of a seed capsule showing a gall, attached to the placenta, in the upper right locule, amongst chaff particles; **B**, young, developing gall (white arrow), attached to placenta, showing ovules connected to it apically (black arrow); **C**, almost fully developed gall with flattened, brown ovules connected to it (arrows); **D**, size and appearance of galls (right) relative to seeds (left) of *Eucalyptus camaldulensis*. Note dark brown, flattened ovules on the straw coloured galls.

Eventually all green tissue is consumed by the larva, and the gall consists of a tough, thin, light-brown wall surrounding a large larval chamber. One or more of the locules of a seed capsule can each contain one or more galls (a maximum of seven galls per seed capsule was recorded), and these may become fused externally, although each retains its own gall chamber.

The galls remain attached to the placenta after the seeds and chaff have been released from the capsule. Adult hymenopterans escape from the galls into the cavity of the capsule through round emergence holes and, from there, either through another hole chewed through the capsule wall, or through the open valves of the capsule (Flock 1957).

No indications were found of a second type of gall that could have been caused by another of the hymenopteran species besides *Q. nova*. Similarly, there was no evidence of the presence of an inquiline, such as a separate chamber in any of the galls, distinct from the one inhabited by the larva of the gall inducer, or of modifications in the morphology of the primary gall.

Juvenile gall-inhabitants and their interactions

The eggs of the gall inducer, *Q. nova*, were identified by dissecting flower buds into which females of this species had been seen inserting their ovipositors. *Quadrastichodella nova* eggs were almost spherical, but with a distinct, long filament. In some instances such an egg was still visible in the centre of a very young gall, but no eggs of this type were found in developed galls. Various eggs that differed from those of *Q. nova* in shape and size were observed in older seed-capsule galls, either attached to live or dead (unidentified) larvae inside the gall, or to the inner walls of galls that contained one or more larvae.

The larvae in galls in the seed capsules were very similar, and more investigation will be required to find ways of distinguishing between the larvae of the various gall-inhabiting species. The only pupae that were easy to distinguish were female pupae of *M. zebrinus*, which could be recognized by the long, exserted ovipositors positioned over the abdomen and reaching forward to the thorax.

In numerous instances, two larvae, or a larva and an egg, were found together in the same gall. Occasionally, the two larvae were of similar size, but usually they differed in size. In a few instances, a living and a dead larva were seen together in a gall, and occasionally the live larva could be seen imbibing fluid from the dead larva. In several galls, a live larva was seen together with the dry, flattened remains of another larva. However, the inability to distinguish between chalcidoid species as juveniles prevented discrimination between parasitoids and hosts.

DNA sequencing

Sequences were amplified for the positively identified adults of *Q. nova*, *M. zebrinus, L. milga* and *Aprostocetus* sp. (GenBank accession numbers: JF800077–JF800090 and KC710350–KC710378) (Dittrich-Schröder *et al.* 2012). A phylogenetic tree (Fig. 3) with high bootstrap support was constructed, linking the unidentified larval specimens to the identified adult specimens and thereby also confirming the taxonomic relationships between the four species involved.

Sequences were also obtained for 14 larvae and two pupae, all of which were matched with the sequences of the identified adults mentioned above (five larvae of *M. zebrinus*, six larvae of *Q. nova*, three larvae of *L. milga* and two pupae of *Aprostocetus* sp.). However, PCR products could not be obtained from any of the eggs, or from specimens that were observed being attacked by another larva, or that had been sharing a gall with another larva.

DISCUSSION

This is the first record of the assemblage of four species of chalcidoid wasps, *Q. nova*, *M. zebrinus*, *L. milga* and *Aprostocetus* sp., co-inhabiting seed-capsule galls of *E. camaldulensis.* Dissections of the seed capsules showed that only one type of gall was present, thereby ruling out the possibility that there could be two gall inducers, or that one of the hymenopterans could be an inquiline. *Quadrastichodella nova* was identified as the only gall inducer in the assemblage by showing that it caused galls to develop when presented with flower buds of the host plant species from which all other insects had been excluded. The other two alleged gall inducers (*M. zebrinus* and *L. milga*), failed to induce galls under these conditions, but *Aprostocetus* sp. has not yet been put to this particular test.

All three species other than *Q. nova* seem likely to be parasitoids, but their exact host relationships remain to be elucidated by further study. The matching of juvenile hymenopterans dissected from the seed-capsule galls with adults of all four hymenopteran species by using molecular methods served as confirmation that all four species indeed developed within the galls, even if the technique has not yet succeeded to discriminate between species being attacked and their attackers. More investigation is required into possible reasons for the failure in replication of the eggs and the larval specimens that were being parasitized. From Fig. 1A and Fig. 1B, it seems plausible that *L. milga* might parasitize the larvae of the gall inducer, *Q. nova*, based on the fact that the two species have the same seasonal emergence pattern, with the former peaking about a month after the latter. In addition, Fig. 2 shows that *Q. nova* emergence numbers were low at sites where those of *L. milga* were high, and *vice versa*, which is typical of the relationship between a host and its parasitoid. There was no evidence that either *M. zebrinus* or *Aprostocetus* sp. had any specific adaptation to attack the remaining two species, due to the unpredictable and mostly low emergences (Fig. 1C, D) of the former two species. One exception to this rule was the relatively high number of *Aprostocutus* sp. adults that emerged at the Rietondale site (Fig. 2). Taking into account that the numbers of *L. milga* were particularly low, and those of *Q. nova* particularly high at this site, one possible explanation could be that *Aprostocetus* sp. was a hyperparasitoid, attacking *L. milga.*

The confirmation that *Q. nova* is indeed a gall inducer in seed capsules of a *Eucalyptus* sp. concurs with both Flock (1957) and Doğanlar & Doğanlar (2008). The reports that the galls in the seed capsules of *E. camaldulensis* are caused by *M. zebrinus* (Grissell 2006) or by *L. milga* (Kim & La Salle 2008) are contradicted by the results of the oviposition trials.

The mistaken belief (Grissell 2006) that *M. zebrinus* was a gall inducer might be ascribed to missing the real gall inducer due to not sampling during the months when its adults are present; to not using molecular techniques to match the larvae in the galls with the adults that emerged, or to the unsubstantiated assumption that the most abundant species emerging from a gall has to be the gall inducer. The locality in South Africa on which the description of *M. zebrinus* was based (Travellers Rest near Clanwilliam), was repeatedly sampled during the course of the current study, and yielded specimens of *Q. nova*, the true gall inducer, in addition to *M. zebrinus* (Table 1). If the *Megastigmus* specimens that were reared from *S. cordatum* are indeed *M. zebrinus*, this would serve as a further reason for doubting that *M. zebrinus* is a gall inducer. Gall inducers need to be intimately adapted to their host plant and, as Grissell (2006) conceded, it seems unlikely that *M. zebrinus* could be sufficiently adaptable to induce galls in two such widely differing plant organs (a dry seed capsule in *E. camaldulensis* and a fleshy drupe in *S. cordatum*). It would be easier to imagine *M. zebrinus* to be a parasitoid of various gall inducers on Myrtaceae trees in general. Its unpredictable seasonal emergence, without any obvious pattern, also suggests that it might parasitize a wider range of hosts. *Megastigmus zebrinus*, as well as other identified or unidentified *Megastigmus* species, have recently been reported as local parasitoids of the native Australian eucalyptus gall wasp, *Leptocybe invasa* Fisher & La Salle (Hymenoptera: Eulophidae) in its invasive range in Israel and Turkey (Protasov *et al.* 2008), India (Kulkarni *et al.* 2010), Thailand (Doğanlar & Hassan 2010; Sangtongpraow & Charernsom 2013), South Africa (Kelly *et al.* 2012), and Brazil (Doğanlar *et al.* 2013).

The original description of *L. milga* was based on emergence data, according to which *L. milga* had been the only species to emerge from seed capsules on one particular tree in South Africa (Kim & La Salle 2008). These data were, however, obtained at a time when the current study had not yet been concluded, before the emergence pattern of the species had been studied. Later during this study, however, when the particular tree (at Rhenish School, Stellenbosch) was visited again, *Q. nova* emerged from the samples, together with *L. milga*.

The current study revealed for the first time that the gall caused by *Q. nova* originates in the placenta of a flower bud, which then becomes transformed and enlarged. This contradicts the suggestions by Bouček (1988), La Salle (1994, 2005) and Kim & La Salle (2008) that the gall is a modified flower bud or seed. Assertions by Doğanlar & Doğanlar (2008) that a gall-like structure was constructed by *Q. nova* larvae by tying together 3–4 young seeds were also shown to be inaccurate.

This paper illustrates the importance of oviposition data, live observation and molecular techniques, in combination with emergence studies and dissection of galls, when assigning biological roles to members of complex gall communities.

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