WASPS (HYMENOPTERA: CHALCIDOIDEA) ASSOCIATED WITH GALLS IN SEED-CAPSULES OF *EUCALYPTUS CAMALDULENSIS* (MYRTACEAE) IN SOUTH AFRICA: SPECIES COMPOSITION, TROPHIC RELATIONSHIPS AND EFFECTS

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DECLARATION

I know the meaning of plagiarism and declare that all of the work in the document, save for that which is properly acknowledged, is my own.

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ABSTRACT

The Australian river red gum (*Eucalyptus camaldulensis* Denhardt) (Myrtaceae) is regarded in South Africa as a valuable general-purpose utility and ornamental tree, as well as an essential source of nectar and pollen to sustain the honeybees which are crucial for pollinating the economically important fruit crops in mainly the Western Cape Province. Despite its utility value, it is regarded as invasive in South Africa, the major concern being the large amounts of water it consumes when growing along watercourses. River red gum is therefore the subject of conflicts in interests between government agencies that target it for control actions, and farmers and beekeepers who utilize it.

As a contribution towards resolving this conflict in interests, a research project was initiated to investigate the potential use of host-specific insects that might reduce the number of viable seeds produced by *E. camaldulensis*. The aim was to reduce its invasive potential while retaining its general utilizability for all concerned. From the start, the need for introduced agents from Australia was weighed against the presence of two species of chalcidoid wasps, both regarded as gall inducers, in the seed capsules of *E. camaldulensis* in South Africa. The current study was motivated by the need for information on the biology of these two species and the effect they were having on seed production in their host plant. As it progressed, three more chalcidoid species, all undescribed at that time and probably of Australian origin, were found to be emerging from the seed capsules of *E. camaldulensis* collected in several parts of South Africa, and these became part of the investigation.

The aims of this study were to determine

- which species was the gall inducer(s),
- the role of each of the other associated hymenopteran species in the gall,
- the extent to which these hymenopteran species affected the reproductive potential of *E. camaldulensis*, and ultimately,
- whether this complex of hymenopteran species, or any single species, had the potential to reduce the invasive potential of *E. camaldulensis* in South Africa, and therefore to serve as biocontrol agents in an integrated management plan of *Eucalyptus* spp. in South Africa.

The first chapter is a literature study, which deals with the possible biological role of each of the five chalcidoid species associated with the galls in the seed capsules of trees in the *Eucalyptus camaldulensis* complex. They are: *Megastigmus zebrinus* Grissell (Torymidae:

Megastigminae); *Quadrastichodella nova* Girault (Eulophidae: Tetrastichinae); *Leprosa milga* Kim & La Salle (Eulophidae: Tetrastichinae); *Aprostocetus* sp. (Eulophidae: Tetrastichinae); and another undescribed species (Eulophidae: Tetrastichinae, genus indet.), which will be referred to as Eulophid #4 and is characterised by a distinct elongate hypopygium. An account is also given of galls, their development and biology, and of gall inducers and other associated insects, e.g. parasitoids, predators and inquilines.

Chapter 2 deals with the geographical distribution of the seed capsule galls of *E. camaldulensis* in South Africa, and with the hymenopteran species that were reared from the samples collected from various parts of the country and during different periods of the year. It also discusses the emergence patterns of the various hymenopteran gall inhabitants, as observed during a detailed, one-year survey of three test trees in different parts of Pretoria. The emergence patterns of the insects are correlated with the phenology of the test trees, as recorded during the same period.

Chapter 3 describes attempts to determine the biological role of each of the five chalcidoid gall inhabitants. This entails the dissection of flowerbuds, flowers and seed capsules of *E. camaldulensis,* oviposition trials in sleeves on intact trees, and the laboratory observation of adults. In some of the cases it was possible to identify the juveniles that were encountered during dissections by matching DNA sequences of the juveniles and of adults. By following all the available leads, it was concluded that *Q. nova* is the primary gall inducer, that *L. milga* probably parasitizes *Q. nova*, and that *M. zebrinus* is a parasitoid of either *Q. nova* or *L. milga*, but that its larvae also feed on gall tissue. *Aprostocetus* is possibly a parasitoid of *L. milga*. The last species (Eulophid #4) was not abundant enough to allow studies of its biology.

In the final chapter, an account is given of the effect of galling on seed production in the three *E. camaldulensis* trees in Pretoria, as an indication of the ability of the gall inducer to reduce the invasiveness of *E. camaldulensis* in South Africa without compromising its valuable attributes. It was concluded that the presence of galls in the seed capsules significantly reduced the number of viable seeds in the capsule. This seed reduction could be expected to reduce the spread of *E. camaldulensis* where it grows along watercourses, which is also where it is most invasive.

This study has expanded the available knowledge on the trophic relationships of chalcidoid wasps associated with eucalypts by showing that:

- the tetrastichine *Quadrastichodella nova* induces galls in the seed capsules of trees of the *Eucalyptus camaldulensis* complex in South Africa;
- the galls develop in the placenta of one of the locules in the seed capsule, and are not modified seeds, or outgrowths from the capsule wall, as previously believed;
- galling of the seed capsules reduces the production of viable seeds in *E. camaldulensis*;
- at least three parasitic chalcidoid species are associated with these galls;
- of these, the tetrastichine *Leprosa milga* probably parasitizes the larvae of the gall inducer;
- the tetrastichine *Aprostocetus* is possibly a hyperparasitoid of *L. milga*;
- the torymid *Megastigmus zebrinus* is a parasitoid that also feeds on gall tissue;
- several authors referring to *M. zebrinus* and *L. milga* as gall inducers have been mistaken.

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INTRODUCTION

South Africa is plagued by many species of invasive alien plants which disrupt ecosytems, deplete the country's water resources, threaten biodiversity and are expensive to control. Predominant among these invaders are perennial trees including *Acacia* and *Eucalyptus* species from Australia and *Pinus* species from North America and Europe (Poynton 1979). The importation of many of these species commenced during the late 19th century, to establish forests of rapidly growing trees to replace the indigenous forests of the southern and eastern Cape which had been over-exploited (Johannsmeier 1993; Richardson *et al.* 2003). This initiative led to continuing introductions of pines and eucalypts under the auspices of the Department of Forestry, and later the Department of Agriculture and Forestry, of the then Union of South Africa Government. Eucalypts were established in all parts of South Africa "as shade trees around farm buildings and railway stations, as woodlots and ornamentals in towns and cities, as small plantations near gold mines on the Highveld, but especially in planted forests in suitable natural-forest regions of the southern Cape, Natal and eastern Transvaal" (Johannsmeier 1993). Different eucalypt species, suited to the diverse climatic regions, were selected and tested (Johannsmeier 1993). One of the prominent species in the mix was *Eucalyptus camaldulensis* Denhardt.

Eucalyptus camaldulensis (river red gum, red gum, river gum, Murray River gum) (fig. 1 A, B, C) is a medium-sized to tall, perennial tree, up to 45 m tall, with a single stem and large bole (Brooker *et al.* 2002). It reaches ages of 500 to 1000 years (Jacobs 1955). The bark on the mid-bole of vigorous, adult trees is deciduous and smooth, the colour varying somewhat with age, peeling off in relatively broad plates (Poynton 1979). The morphology of the tree varies considerably throughout its range, and a number of intraspecific taxa have been described, the most widespread of which is *E. camaldulensis* var. *camaldulensis* (West undated).

In Australia, *E. camaldulensis* is the most widely distributed of all eucalypts (Poynton 1979; Chippendale & Wolf 1981; Chippendale & Johnston 1983). It grows naturally in every state of mainland Australia, spanning the continent from north to south and from east to west. It occurs throughout most of the dry inland, though almost invariably along water-courses or on floodplains subject to frequent or periodic inundation (Poynton 1979; Costermans 1989; Brooker *et al.* 2002). It frequently dominates riparian communities, particularly in the Murray-Darling catchment (West undated), and commonly forms ribbon stands, but

sometimes extends over large areas of regularly flooded flats (Cunningham *et al.* 1981), forming pure open forests or woodlands (Costermans 1989).

Eucalyptus camaldulensis (as *E. rostrata*) was included in a list of trees recommended for planting in the Cape Colony in terms of Act No. 4 of 1876 (Storr Lister 1884). Poynton (1979) reported that between 1893 and 1972, more than 50 seedlots of *E. camaldulensis* were introduced into South Africa, most of which were assumed to have come from southern New South Wales or Victoria, although their exact origin is unknown. In the early days, seed of this species was almost invariably sourced from the Murray-Darling River Basin to establish trials, and for afforestation outside Australia (Poynton 1979), and since *E. camaldulensis* var. *camaldulensis* is the only intraspecific taxon occurring in the Murray-Darling Basin (West undated), most of the trees in South Africa are likely to belong to this variety. Due to the extensive area covered by this Basin, from which the seed was apparently introduced, combined with the natural intraspecific variability of the species in Australia, *E. camaldulensis* in South Africa is extremely variable. To complicate the matter further, it is difficult to distinguish between pure *E. camaldulensis* (river red gum), pure *E. tereticornis* Smith (forest red gum), and hybrids between these two species in South Africa.

The growth form (fig. 1 A) and vigour of *E. camaldulensis* soon proved to be inferior to those of the main commercial species in South Africa, and currently it plays hardly any role in commercial forestry. Only in areas where no better alternatives exist, e.g. in the drier areas of the country, is it still grown commercially (Poynton 1979), in addition to its use in breeding programmes where it can be crossed with *E. grandis* to obtain a hybrid that is more drought resistant than *E. grandis* but that grows faster than *E. camaldulensis* (Van Wyk 1994; Dye 1996).

Despite its trivial role in forestry, *E. camaldulensis* is one of the most dependable general purpose utility trees in the dry parts of South Africa, where it is used mainly in farm woodlots, shelterbelts (fig. 1 E) and avenues, and as a shade tree (Poynton 1979; Haigh 1993). Though not as drought- or frost-resistant as some of the other eucalypts, it endures the combined effect of drought and frost relatively well (Poynton 1979). It is also a highly sought after ornamental tree (Poynton 1979).

Eucalypts, including *E. camaldulensis*, are of considerable importance to beekeepers in South Africa as a source of forage (fig. 1 D), which ensures the survival of honeybee

Figure 1. River red gum, *Eucalyptus camaldulensis*, in South Africa: identification, value and threats. A: Smooth bark and drooping leaves. B. Beaked flowerbuds, off-white blossoms and reddish shoots. C. Seed capsules with exserted valves. D. Honeybees foraging on flowers. E. Planted as shelter strip on farm. F. Inland infestation: all seedlings of same age group. G. Dense infestation along Olifants River.

colonies during winter; to maintain healthy and strong colonies throughout the year; and to provide beekeepers with an income from honey (Johannsmeier 1993; Johannsmeier & Mostert 2001; Allsopp & Cherry 2004). The value of gum trees to beekeepers lies in their dependability, regular flowering, and constant nectar secretion and pollen production, which cannot be equalled by indigenous South African vegetation, crops or weeds (Johannsmeier 1993; Allsopp & Cherry 2004). Although the single most important honey source in South Africa is *E. grandis* Hill ex Maiden, which is grown mainly in the high-rainfall regions of Mpumalanga and KwaZulu-Natal (Johannsmeier 1993), in the Western Cape *E. camaldulensis* is regarded as the second most important eucalypt, after sugar gum (*E. cladocalyx* F. Mueller) (Allsopp & Cherry 2004). The major importance of honeybees in South Africa lies, however, not in the production of honey, but in their role as pollinators of crops. Honeybees pollinate at least 26 crops (including the entire pome and stone fruit crop) in the Western Cape Province, which in 2004 were earning about R5 billion in exports per year and supporting 170 000 employees (Allsopp & Cherry 2004). To achieve this, strong honeybee colonies are needed early in spring; the presence of eucalypts throughout the country is the only way in which this can be ensured. The value of bee-pollinated crops and earnings from paid pollination far outweigh the income from honey crops (Johannsmeier 1993). The eucalypts on which the honeybee colonies rely for their survival, only rarely belong to the owners of the hives, but mostly grow on the property of other farmers in the vicinity, or on municipal or provincial land (Allsopp & Cherry 2004).

There have been prolonged debates about whether eucalypts in South Africa are invasive and, if so, which of the species are invasive, and under which circumstances they become invasive. Richardson *et al.* (2000) defined invasive plants as naturalised plants that produce reproductive offspring, often in very large numbers, at considerable distances from parent plants, and thus have the potential to spread over a considerable area (e.g. more than 100 m in less than 50 years, for taxa spreading by seed). It should therefore be possible to obtain a reasonable objective answer to the above questions, yet these required figures are still not available for eucalypts in South Africa.

Eucalypts in general have been described as very poor invaders, seldom spreading considerable distances from planting sites in South Africa, and frequently regenerating only sporadically (Forsyth *et al.* 2004); seldom being able to compete with parents under plantation conditions, but being able to invade pine plantations and denuded areas (Poynton 1979; Allsopp & Cherry 2004). Information on *E. camaldulensis* in particular occurs very

rarely in the body of literature dealing with plant invasions in South Africa, especially prior to 2000. Richardson *et al.* (1997) failed to mention *E. camaldulensis* in a compilation of major invaders in the fynbos biome, or in their list of 84 important environmental weeds in southern African biomes.

One of the first published references to *E. camaldulensis* as an invader was in a guide to declared weeds and invaders in South Africa (Henderson 2001) that accompanied the first listing, during March 2001, of various *Eucalyptus* species by the Conservation of Agricultural Resources Act 1983 (Act No. 43 of 1983) (commonly referred to as CARA) (Anonymous 2001). In the scientific literature in South Africa it was also first recorded by Henderson (2002), who mentioned its ability to form extensive stands and use large volumes of water along watercourses (fig. 1 G). In a later paper, Henderson (2006) recorded *E. camaldulensis* as a transformer (an invasive plant with the potential to transform ecosystems), occurring in 127 quarter-degree squares (a division of the country into blocks which cover 25 minutes of longitude by 25 minutes of latitude) with a calculated rate of spread of 1.187 quarter-degree squares per year, distributed mainly in the dry interior, but with extension into more mesic areas. It was, however, not recorded among the top ten species in any of the biomes (Henderson 2007). A rapid assessment in the Western Cape and Mpumalanga, South Africa, of the invasive status of some of the *Eucalyptus* species listed in the CARA regulations, was published by Forsyth *et al.* (2004). This involved spot surveys, based on visual observations, along stretches of the Sonderend, Berg and Olifants rivers in the Western Cape and in the catchments of the Sabie and Crocodile rivers in Mpumalanga, and species were classified as invasive in accordance with the definitions by Richardson *et al.* (2000). The authors regarded *E. camaldulensis* as the greatest threat, based on its distribution throughout much of South Africa and the fact that it had already transformed long stretches of rivers and dam shores. They referred to it as a major environmental weed, which had been underestimated in previous reviews of alien plant invasions in South Africa. Allsopp & Cherry (2004) agreed that *E. camaldulensis* dominated river-courses and was highly invasive all along the Berg River, and in many parts of the Breede River.

Despite references (Henderson 2002) to the high water usage by *E. camaldulensis* where it grows along watercourses in South Africa, not a single scientific study could be found that investigated and quantified the effect of this species, specifically, on water runoff in South Africa. Yet water usage was the major motivation for the inclusion of *E. camaldulensis* and other eucalypt species in the CARA list of declared weeds and plant invaders, in addition to

providing the mandate for the *Working for Water* Programme to clear the CARA-listed eucalypt species in parts of the country (Van Wilgen *et al.* 2002). The widely quoted publication of Versfeld *et al.* (1998), indicating that some 7% (3300 million m^3) of South Africa's mean annual runoff was lost through transpiration by woody alien plant species invading catchments, riparian zones and wetlands, did not mention *E. camaldulensis* by name. It is widely recognised that afforestation reduces runoff water yield, through changes in transpiration, through the interception of precipitation by the canopy, and through evaporation of this intercepted water (Van Lill *et al.* 1980; Dye 1996; Le Maitre *et al.* 1996; Jewitt 2002; Görgens & Van Wilgen 2004; Farley *et al.* 2005), especially when grasslands and shrublands are replaced by tall, evergreen forests (Farley *et al.* 2005). *Eucalyptus* species are known to have particularly high transpiration rates, due to the fact that they develop deep roots at an early age, grow rapidly early in their life, undergo canopy closure, are able to reach deep ground water, and, being evergreen, lack the period of senescence that characterises most crops and grasslands (Farley *et al.* 2005; Calder & Dye 2001). However, since *E. camaldulensis* is not a commercial forestry species and is not planted in catchment areas in South Africa, it does not feature in any of the published catchment experiments for South Africa (Nänni 1971; Van Lill *et al.* 1980; Dye 1996), and there is no evidence to suggest that the water usage by *E. camaldulensis* is similar to that of the more frequently monitored *E. grandis*. Being an invader of riverine, rather than of catchment areas in South Africa, *E. camaldulensis* has access to large quantities of water, which might translate into larger streamflow reductions (Dye 1996). Conversely, Calder & Dye (2001) indicated that eucalypts growing in moist areas cause a smaller decrease in water runoff, in both absolute and percentage terms, than in water limited conditions in dry areas. For the riverine situations, which are the areas most commonly invaded by *E. camaldulensis*, no records could be found of any water usage studies that have been carried out in South Africa. Anecdotal evidence was provided by Cambray (2006) on the drying up of the Kariega River in the Eastern Cape Province, where a 15 km stretch of river was entirely overgrown with *E. camaldulensis* in combination with *Acacia cyclops*, causing the river to stop flowing altogether.

It has been shown in eastern Ethiopia that woodlots of *E. camaldulensis* have the potential to deplete nutrients in adjacent crops, pose serious root competition and have an allelopathic effect on the crops (Gindaba *et al.* 2007), although similar effects have not been demonstrated in South Africa.

Invasions of eucalypts in general are believed to increase the fire hazard through their high fuel load, thereby increasing fire intensities. The wildfire in the Cape Peninsula during March 1999 was generally blamed on the presence of invasive alien species, including eucalypts, although *E. camaldulensis* has not been mentioned by name. Such intense fires can cause severe damage to soils, followed by erosion (Van Wilgen *et al.* 2002).

In the public mind, the value of alien forestry species, including eucalypts, has, until quite recently, overshadowed the threats the trees pose to the ecology, water resources and biodiversity of South Africa. The forestry industry itself was aware of the ability of plantations to reduce the water runoff in afforested water catchments, and had its own regulations to restrict afforestation near watercourses (Dye 1996). However, general awareness of the negative attributes especially of woody alien vegetation was only created by the *Working for Water* (WfW) Programme, an initiative of the Department of Water Affairs and Forestry. Its inception in 1995 was based on research that demonstrated the current and potential impacts of invasive alien trees and shrubs on water resources in South Africa, and the economic benefits of intervention. By controlling invasive alien plants the intention is to protect water resources and ensure security of water supplies (Van Wilgen *et al.* 2002). Its clearing operations are conducted in a labour-intensive manner, doubling as a public-works programme aimed at poverty alleviation (Macdonald 2004). A large proportion of WfW's first clearing operations were centred in the Western Cape Province, targeting woody alien vegetation (mainly Australian *Acacia* and *Eucalyptus* species growing in riverine situations, and *Pinus* and *Hakea* species in mountain catchments) (Marais *et al.* 2004). Being an invasive riverine species, *E. camaldulensis* would have been one of the important targets for clearing.

As with other important commercial species that become invasive, the control of *E. camaldulensis* in South Africa was, and still is, fraught with conflicts of interests (Van Wilgen *et al.* 2002). Although *E. camaldulensis* is not an important forestry species, the major concern is the effect that such clearing could have on the honeybees that pollinate deciduous fruit trees, especially in the Western Cape Province (Allsopp & Cherry 2004). The regulations in terms of CARA recognise a category of invasive species (designated Category 2), into which *E. camaldulensis* falls, and which allows land users to register demarcated stands they regard as valuable, including those for beekeeping. This brings with it the responsibility for preventing the plants from spreading, and legislates that all specimens growing in riverine situations should be removed (Anonymous 2001). Less than 1% of

beekeepers own the land on which their bee colonies forage, and they were concerned that, with the promulgation of the legislation, landowners would opt for removing the trees rather than go through the process of demarcation, paying water tax and maintaining the required control measures (Allsopp & Cherry 2004). The Western Cape Bee Industry Association (WCBA) therefore appealed to the Minister of Agriculture to reconsider the regulations regarding eucalypts (letter by J.D. Smit, Chairman: WCBA to Minister of Agriculture, dated 6 June 2001). As an interim measure it was suggested that clearing of "Category 2" eucalypts outside demarcated areas be restricted to only those specimens that were growing in or near watercourses, wetlands and other ecologically sensitive areas. Meanwhile, research into the invasiveness of different eucalypts in South Africa, and into the viability of planting alternative food plants to sustain honeybee colonies, should be funded.

During 2002, WfW and the South African Bee Industry (SABIO) agreed to have a survey undertaken that should determine the importance of eucalypts to the beekeeping and related industries, and to develop an operational strategy regarding the removal of eucalypts (Allsopp & Cherry 2004). Some of the findings of this survey were that honeybees spent 76% of all "colony months" on eucalypts; that 66% of all honey was produced from eucalypts (15% of which was produced on *E. camaldulensis*), and that the direct monetary value (in terms of honey production) of the seven CARA-listed eucalypt species in the Western Cape was between R6.5 million and R7.0 million per annum. Of the 50 000 pollination events that were required by the fruit industry per year, 96% were provided by colonies utilizing CARA-listed eucalypts. Of these, *E. cladocalyx* (sugar gum) was responsible for 87% of pollination, while *E. camaldulensis* and *E. conferruminata* (at that time referred to as *E. lehmannii* in South Africa) together were responsible for 59% of the remainder. The total cost to growers, if all seven eucalypt species listed in the 2001 version of CARA were to be removed, was calculated at R113.4 million per annum. The losses might be reduced to R66.9 million if only *E. camaldulensis* and *E. conferruminata* (which were assumed to be the most invasive and ecologically harmful of the seven listed eucalypt species) were to be removed (Allsopp & Cherry 2004). The report recommended an investigation into the nature of the different CARA-listed eucalypt species and the threats they posed to the environment. It was stated that *E. camaldulensis* could be excluded from such an investigation - probably since its invasiveness and the threat it posed to water resources were not being disputed (Allsopp & Cherry 2004).

During a rapid assessment of the invasive status of *Eucalyptus* species in the Western Cape and Mpumalanga Provinces in South Africa, mentioned earlier (Forsyth *et al.* 2004), it was concluded that the three most invasive species in the country were *E. conferruminata* (Bald Island marlock) (as *E. lehmannii*, commonly known as spider gum), *E. camaldulensis* and *E. grandis* (saligna gum). The authors suggested that all *Eucalyptus* species growing in riparian areas and nature reserves be cleared, but that only *E. conferruminata, E. camaldulensis* and *E. grandis* should also be targeted for clearing in other situations, outside areas demarcated for their cultivation.

Mindful of the necessity of supplementing the conventional management practices by biological control, to ensure that cleared areas did not become reinvaded, WfW became a major funder of research into the biological control of invasive alien plants in South Africa (Van Wilgen 2004; Zimmermann & Neser 1999; Zimmermann *et al.* 2004). As part of this investment, WfW contracted the Plant Protection Research Institute of the Agricultural Research Council (ARC-PPRI) to carry out research into the biological control of three invasive *Eucalyptus* species: *E. camaldulensis*, *E. cladocalyx* and *E. conferruminata*. These three species were probably selected by WfW from the seven *Eucalyptus* species listed in the CARA regulations because they were believed to be the most problematic in the Western Cape Province, in addition to not being commercial forestry species. WfW specified that the research should take account of potential conflicts of interest and therefore focus on seed-destroying agents only.

The use of biocontrol agents that reduce the aggressiveness and spread of the invasive plant without affecting its useful properties was one of several tactics that were identified by Neser & Moran (1985) for evading conflicts of interest. This might be achieved by using insects that attack seeds, or that prevent seeding. South African researchers demonstrated that seed-attacking organisms had previously been underestimated as a factor in suppressing the invasive success of plants in a new environment, and that their use was an important option in the control of invasive alien plants (Neser & Kluge 1986). This type of biological control has been used with considerable success in South Africa in the past in cases where alien tree species were still being utilized for their timber, fodder or shade value, but where the prolific seed production caused the species to become invasive (Neser & Kluge 1986; Hoffmann 1991; Van Wilgen *et al.* 2002). Examples of successful (mainly South African) biocontrol projects based on organisms that reduce flowering were described by Dennill & Donnelly (1991); Hoffmann & Moran (1991); Morris (1991); Dennill *et al.*

(1999); Hoffmann & Moran (1999); Morris (1999) and Seastedt *et al.* (2003). Projects utilizing organisms that cause the destruction of seeds were published by Dennill & Donnelly (1991); Hoffmann & Moran (1991); Kluge & Neser (1991); Zimmermann (1991); Dennill *et al.* (1999); Gordon (1999); Hoffmann & Moran (1999), Impson *et al.* (1999); Impson *et al.* 2000; Impson & Moran (2003); Donnelly & Hoffmann (2004) and Impson *et al.* (2004).

Eucalyptus camaldulensis, which has the widest distribution of all eucalypts in South Africa and is the most troublesome of the three species selected by WfW as priority species for control, was selected by ARC-PPRI as the first target plant for research. In planning the selection of guilds of natural enemies for introduction as potential biocontrol agents, the potential conflicts in interests were kept in mind. The value attached to *E. camaldulensis* as a utility tree in South Africa precluded the introduction of natural enemies that, by attacking the vegetative parts of the plant, had the potential to kill or weaken the trees. Similarly, flowerbud-feeding or flower-feeding natural enemies, which would reduce the value of the trees as a source of nectar and pollen for honeybees, had to be ruled out as potential biocontrol agents. The only parts of the tree that were regarded as expendable in all situations were the seed capsules and seeds, and these were therefore targeted for potential biological control. It was argued that a reduction in seeds would contribute towards reducing the invasive potential of the plants while still allowing the utilization of their nectar, pollen, wood, and shelter. It is true that the dense stands of *E. camaldulensis* trees lining some of the most important river systems in the Western Cape would still need to be controlled by some other means, e.g. killing them by ringbarking. However, the biological control of their seeds could be expected to reduce the number of seedlings that would still germinate, thus reducing the effort required in follow-up clearing, and to limit their spread.

According to protocols for the biological control of invasive alien plant species in South Africa (Holtzhausen & Bolton 1994), a survey or investigation of the target weed in its adopted country is required, to determine whether any insects and pathogens have colonised the target weed. If such phytophagous species are found, their potential as biocontrol agents should be investigated. It is important that the presence of such organisms in the country, as well as their effect on the target weed, are known before the introduction of further natural enemies from the weed's native range is considered. It is possible that these insects or pathogens will be effective in controlling the target weed on their own, or can be augmented to be made more effective in one of several ways, in which

case no further biocontrol candidates would need to be introduced. Alternatively, their effect might be significant, but not sufficient to suppress the invasive potential of the target weed to a level below the economic or environmental threshold level. In such a case, natural enemies from a different feeding guild might have to be introduced from the weed's native range to supplement the insects or pathogens already present in the extended range of the host plant. Having a comprehensive understanding of the natural enemies associated with the plant species could prevent the unnecessary consideration of the same or similar species as biocontrol candidates, in which case the project would still be a scientific success (Myers & Bazely 2003). In each of the above scenarios, the local surveys will have saved the research organisation time and funds, and will have avoided adoption of a "lottery approach" (Myers 1985; McEvoy & Coombs 2001) to the biological control programme (i.e. the continuous introduction of species of agents in the hope that one will become effective in the appropriate location). A pre-introduction investigation of natural enemies already on the target weed in the country of introduction therefore ensures that only necessary and potentially effective agents are introduced into the country. The knowledge obtained by such studies is also potentially valuable in future research projects involving closely related target weed species.

When a survey of *E. camaldulensis* was initiated, the National Collection of Insects in Pretoria was found to already have records of two species of chalcidoid wasps – a torymid and a eulophid - that were associated with the seed capsules of *E. camaldulensis* in South Africa (see chapter 1). One of these was known from the literature to be a gall inducer (*Quadrastichodella nova*) (Flock 1957; Timberlake 1957), while a paper describing the second species (*Megastigmus zebrinus*) as another gall inducer (Grissell 2006) was in preparation (see chapter 1).

It had been known since the 1980s that *Megastigmus* species were emerging from eucalypt seed capsules in Australia (S. Neser, ARC-PPRI, pers. comm. 2004). This was regarded as an indication that it would be worth undertaking an investigation into the potential of seed-attacking insects to reduce the invasive potential of eucalypts. A pilot study in South Africa during 2000 and 2001, carried out by R. Adair (then ARC-PPRI) & L. Madire (ARC-PPRI), indicated the presence of an undescribed *Megastigmus* sp. as well as unidentified hymenopterans associated with the seed capsules of *E. camaldulensis* and *E. tereticornis* in this country (R. Adair, then ARC-PPRI & L. Madire, ARC-PPRI, pers. comm. 2004). Emergence holes were also found in capsules of *E. blakelyi* and *E. tereticornis* (R. Adair & L.

Madire pers. comm. 2004). The existence of specialized seed-feeding insects (as they were considered to be at that time) on *Eucalyptus* was regarded as an indication that biological control of targeted species in this genus was possible.

The existence of at least two species of chalcidoid wasps, both regarded as gall inducers in the seed capsules of *E. camaldulensis* in South Africa, and the lack of information on the effect that they were having on seed production in their host plant, provided the impetus for this study. During the course of this project, three more chalcidoid species, all undescribed at that time, were found to be emerging from the seed capsules of *E. camaldulensis* collected in several parts of South Africa, and these have also been included in the study.

The aims of this project were therefore to determine:

- the identity of the gall inducer(s),
- the role of each of the other associated hymenopteran species in the gall,
- the extent to which these hymenopteran species affect the reproductive potential of *E. camaldulensis* and, ultimately,
- whether this complex of hymenopteran species, or any single species, is able to reduce the invasive potential of *E. camaldulensis* in South Africa, and therefore to serve as biocontrol agents in an integrated management plan of *Eucalyptus* spp. in South Africa.

CHAPTER 1: INSECT SPECIES ASSOCIATED WITH GALLS IN THE SEED CAPSULES OF THE *EUCALYPTUS CAMALDULENSIS* COMPLEX IN SOUTH AFRICA

1.1 INTRODUCTION (Fig. 1.1)

This study confirmed that five hymenopteran species are utilising the capsules of *Eucalyptus camaldulensis* in South Africa, including three species not previously recorded. The five species are:

- *Megastigmus zebrinus* Grissell (Torymidae: Megastigminae) (fig. 1.1 A-E)
- *Quadrastichodella nova* Girault (Eulophidae: Tetrastichinae) (fig. 1.1 F, G)
- *Leprosa milga* Kim & La Salle (Eulophidae: Tetrastichinae) (fig. 1.1 H-J)
- *Aprostocetus* sp. (Eulophidae: Tetrastichinae) (fig. 1.1 K, L)
- An undescribed species (Eulophidae: Tetrastichinae, genus indet.), henceforth referred to as Eulophid #4.

Of these, only *Q. nova* had been described before the project was initiated; *M. zebrinus* was described during 2006 and *L. milga* during 2008, by which time the research had almost been concluded.

The potential of the seed capsule galler in curbing seed production in *E. camaldulensis* could only be examined once it was known which of the species was the gall inducer. The first step was therefore to determine which one (or more) of the five hymenopteran species induced the galls, and what the relationship of each of the remaining species was to the gallformer. They could be inquilines (see Part II of Chapter 1); predators or primary parasitoids, which would reduce the populations of the gallformer or inquilines; or hyperparasitoids, which would reduce the numbers of primary parasitoids.

The biology of an insect can often be inferred by studying biological records of its closest relatives. In the case of gall associates, useful clues might be obtained from the literature regarding the behaviour and interrelationships of different members of the communities in various galls, to enable the correct interpretation of observations made during the planned dissection of *E. camaldulensis* galls. In addition, literature on similar galls in related host plants might be studied, as gall-associates often co-evolve and speciate with their host plants.

This chapter presents a literature review of the recorded characteristics and biology of the five members of the insect community associated with the seed capsule galls in the *E. camaldulensis* complex, including the groups to which they belong. This is followed by a discussion of the potential roles of insects in plant galls.

1.2 LITERATURE STUDY OF THE GALL INHABITANTS AND THEIR RELATIVES

All five members of the gall community under investigation belong to the superfamily Chalcidoidea. Most chalcidoid species are entomophagous and, indeed, this superfamily includes the majority of all entomophagous insects (Clausen 1962). Of these, most are parasitoids, but a number of species with predaceous larvae are also known (Noyes 2003). Some chalcidoid families, including Torymidae and Eulophidae, contain phytophagous species (Bouček 1988) and, particularly in Australasia, some chalcidoid species are known and others are suspected to be gall-makers of mainly eucalypts and acacias (Bouček 1988). Austin *et al.* (2004) pointed out that "one of the more interesting radiations among Australian parasitoids (was) that of gall induction in the Chalcidoidea".

Three of the species associated with the seed capsule galls in the *E. camaldulensis* complex (*M. zebrinus*, *Q. nova* and *L. milga*) appear in the literature as being able to induce galls in the seed capsules of eucalypts.

1.2.1 *Megastigmus zebrinus* Grissell, 2006 (Torymidae: Megastigminae)

Torymidae usually have relatively short life cycles, some of less than a month, but this does not always result in the expected number of generations each year, with many species entering a quiescent phase during their mature larval stage and some having only one generation per year (Clausen 1962). Torymid eggs are elongate-oval to kidney-shaped, with the anterior end being the broadest and terminating in a short, rounded protuberance; the posterior end is somewhat attenuated, sometimes terminating in a sharp point (Clausen 1962). The larvae are hymenopteriform, (i.e. featureless with a pale body, head capsule weakly developed or absent, body spindle-shaped, without thoracic legs (Gordh & Headrick 2001)), with large cylindrical antennae and sensory setae on the head, as well as heavy and long sensory setae on the body segments and a band of short setae on each segment. The larval mandibles can be simple, tridentate or 4-dentate (Clausen 1962).

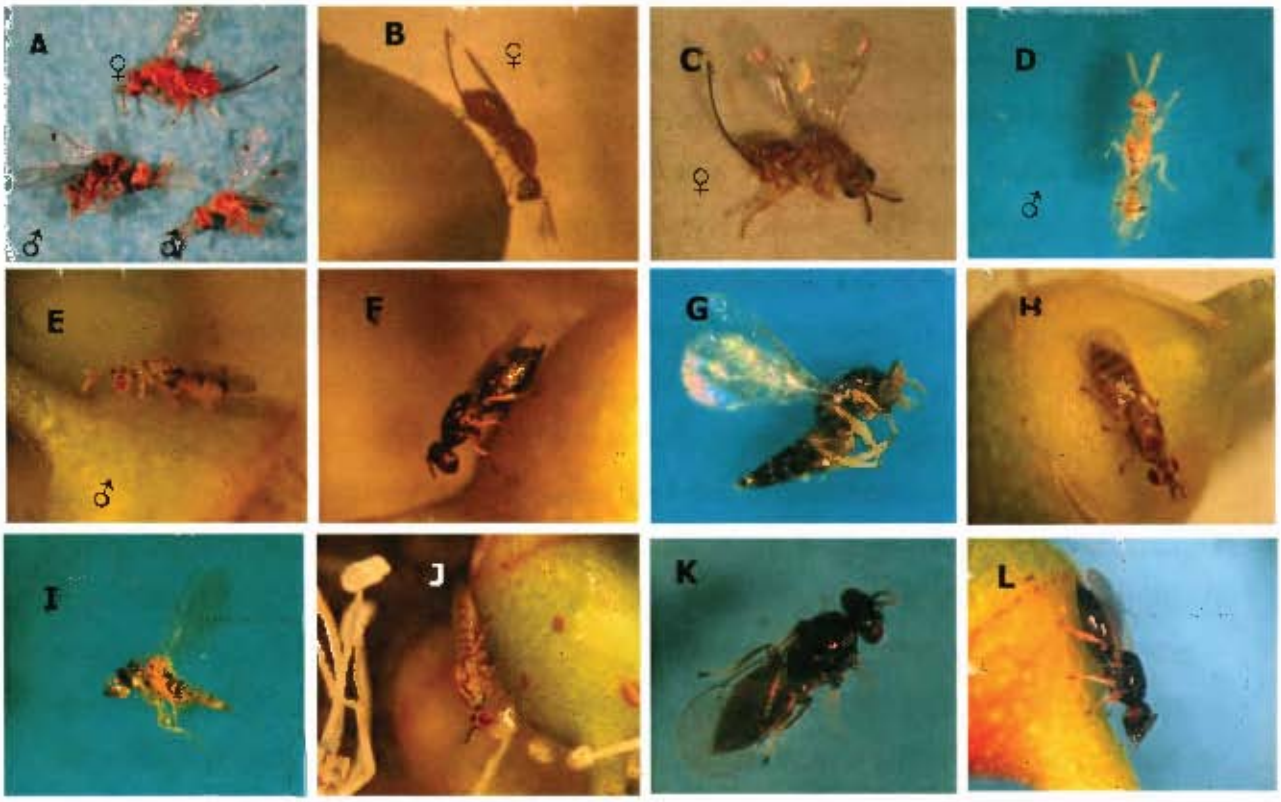


Figure 1.1. Four of the five hymenopteran species forming part of the species complex in galled seed capsules of the *Eucalyptus camaldulensis* complex in South Africa: A-E *Megastigmus zebrinus* (A female and two morphological types of males; B,C females; D,E males); F,G *Quadrastichodella nova*; H-J *Leprosa milga* (J female feeding on pollen); K,L *Aprostocetus* sp.

Within the subfamily Toryminae, many *Torymus* species are parasitoids of gall insects and some are facultative parasitoids, which develop on a host larva as well as phytophagously in gall tissue (Grissell 1976).

Approximately one-third of the species within the subfamily Megastigminae are phytophagous in seeds, one-third are entomophagous (the majority on gall-forming hosts), and for the remainder the hosts (and therefore the biology) are unknown (Grissell 1999, 2006). Roughly half of the taxa in the subfamily occur in Australia, and about 80% of the members belong to the genus *Megastigmus* Dalman, 1820 (Grissell 1999). Bouček (1988) noted that, in the northern hemisphere, the entomophagous *Megastigmus* species differed from the seed-feeding ones in having metallic gloss, but that a similar distinction was not yet known for Australian *Megastigmus* species.

Grissell (1976, 1999, 2006) referred to the paucity of biological information for the genus *Megastigmus*, but noted that approximately half of the 126 *Megastigmus* species were obligate seed feeders, feeding within a single seed embryo, mostly in the plant families Pinaceae and Rosaceae (a small number of Old World species developing in seeds of the

Anacardiaceae were also mentioned by Grissell & Prinsloo (2001); Scheffer & Grissell (2003); and La Salle (2005)). The other species were either partial phytophages (also known as facultative parasitoids) (in which the larva feeds first on plant tissue and later consumes the gall-forming host), obligate egg-larval parasitoids (in which the egg is laid in the egg of a gall-forming host but does not hatch until the host larva has achieved a certain size) or larval parasitoids of gall-forming Hymenoptera, inquilines, or gall-formers (Grissell 1999). Thirty-six *Megastigmus* species had been reared from galls on several plant species in Australia. Grissell (1999) alluded to one known gall-forming *Megastigmus* species, supported by "unpublished personal observation based on dissections". His description during 2006 of *M. zebrinus* as the first *Megastigmus* species known to be a gall-inducer, from material collected during 1998 (see next paragraph), supports the assumption that he was referring to *M. zebrinus*. La Salle (2005) regarded it as a distinct possibility that the genus *Megastigmus* might contain gall inducers, since it already contained seed feeders, parasitoids of gall inducers and possibly inquilines. One case is known (Von Aderkas *et al.* 2005) of a *Megastigmus* species (*M. spermotrophus* Wachtl.) that prevents the normal abortion of infested megagametophytes of unpollinated ovules of the Douglas-fir and, instead, causes the plant to respond as if it were feeding its own embryo by accumulating storage products, which are then consumed by the larva. However, this cannot be regarded as a typical gall, because the presence of the insect does not alter the normal tissue structure to produce nutritional cells.

Megastigmus zebrinus Grissell was first recorded during January 1998 from galled seed capsules of *E. camaldulensis* at Traveller's Rest near Clanwilliam in the Western Cape Province, South Africa (Grissell 2006). From the subsequent discovery of *M. zebrinus* in a natural stand of *E. camaldulensis* near Condamine River, Australia, he deduced that the species was of Australian origin. From the same sample of *E. camaldulensis* seed capsules in Australia, he also reared an unknown species, probably undescribed, that was somewhat similar in appearance to *M. zebrinus.* Grissell (2006) found various developmental stages of (what he presumed to be) *M. zebrinus* in seed-like galls within the seed capsules of *E. camaldulensis* from South Africa, with no other indication of gall associates. From this he concluded that *M. zebrinus* had caused the galls, which would make it the first *Megastigmus* species known to be a true gall inducer. Further confirmation of this conclusion was the fact that he had found no other gall associates in the galls containing larvae or pupae of *M. zebrinus*, nor any other hymenopteran species amongst the hundreds of *M. zebrinus* adults

that he swept in the vicinity of the tree from which he first collected the galled seed capsules of *E. camaldulensis* (Grissell 2006).

Specimens of *M. zebrinus* were reared shortly thereafter (February 1998) by S. Neser (ARC-PPRI) from seed capsules of the *E. camaldulensis* complex: in the North West Province near Buffelspoort (25.51S 27.24E) along with *Quadrastichodella nova*, and in March 1998 in the Western Cape Province from Somerset West (34.05S 18.51E) (The Chalcidoidea Specimen Database of the Biosystematics Division, ARC- Plant Protection Research Institute, Pretoria).

Surprisingly, *M. zebrinus* was also reared during June 1998 from the drupes of a cultivated specimen of *Syzygium cordatum* Hochstetter ex Krauss (Myrtaceae), in Cape Town (Western Cape Province, South Africa) by S. van Noort (Grissell 2006). This was confirmed by Dr Simon Van Noort (Curator of Entomology, South African Museum, Cape Town, pers. com., 28/10/2004) as well as the National Collection of Insects (ARC-PPRI) in Pretoria, which holds specimens of *M. zebrinus* that were reared during February 1998 from *S. cordatum* fruit collected around Cape Town by J.J. Cillie. The *M. zebrinus* specimens reared from *S. cordatum* differed slightly morphologically from those reared from *E. camaldulensis*, but molecular data proved them to be one species (Grissell 2006).

1.2.2 *Quadrastichodella nova* Girault, 1922 (Eulophidae: Tetrastichinae) (Table 1.1)

Eulophids have relatively short life cycles, mostly between 15 and 25 days (Clausen 1962), which would allow for a considerable number of generations per year. However, most species have only two or three generations per year, or just a single annual generation, corresponding to that of their host. They usually enter a quiescent phase as mature larvae in the body of their host or in a gall, while a few become quiescent as pupae or adults. Practically all species in the Tetrastichinae have a sex ratio heavily biased in favour of females (Clausen 1962); some tetrastichines are thelytokous, which means that they are able to produce diploid females asexually (Calsuen 1962; Naumann 1991).

The eggs of most eulophids are simple, oblong or ovate, and often slightly arched (Clausen 1962). Eulophids have between three and five larval instars; the first-instar larvae are hymenopteriform and somewhat cylindrical, but they lack unique characteristics by which they can be readily distinguished from related families (Clausen 1962). The mature larvae

are usually simple in form, having very few integumentary spines or setae and usually lacking surface sculpturing. The larval mandibles are usually simple (Clausen 1962).

Most eulophids are primary parasitoids, some are hyperparasitoids (Bouček 1988), and a few species induce plant galls (Clausen 1962). Over half of the 328 eulophid genera were recorded from Australia (Bouček 1988). Tetrastichines are mainly primary parasitoids of the immature stages of a variety of insects (Clausen 1962), with ectoparasitism being widespread within the subfamily (Bouček 1988). At least one tetrastichine species (*Aprostocetus* sp.) living as a larval predator of nematodes is on record (Van den Berg *et al.* 1990); another tetrastichine species (*Tetrastichus eriophyes* Taylor) has been recorded as a predator of eriophyid mites (Monaco 1971). Many Australian members of the Tetrastichinae parasitise galls (Naumann 1991). Two categories of tetrastichines contain gall-inducers: Australian gall inducers associated with Myrtaceae (*Oncastichus*, *Ophelimus*, *Epichrysocharis*, *Quadrastichodella*, *Leptocybe*, *Moona* and *Leprosa*), and a Neotropical category containing genera such as *Paragaleopsomyia* and '*Exurus*', which may or may not include gall-inducing species (La Salle 2005; La Salle *et al.* 2009 a & b). The genus *Selitrichodes* has also recently been found to contain a species, *S. globulus*, which induces galls on *Eucalyptus globulus* (La Salle *et al.* 2009a). A few tetrastichines, including *Aprostocetus* spp., which do not fit into either of the two categories, are also thought to be gall-inducers (La Salle 2005). One exclusively phytophagous genus, *Anselmella*, is known (Bouček 1988). Ferraz & Monteiro (2003) recorded two tetrastichine species living as inquilines in cecidomyid galls.

Quadrastichodella Girault, 1913, is an Australasian genus (Bouček 1988; La Salle 1994a), with six recognized species (Ikeda 1999), considered to be phytophagous (Bouček 1988) and associated with various *Eucalyptus* species (La Salle 1994a; Kim *et al.* 2005; Kim & La Salle 2008); one known exception (*Q. gracilis*) is associated with *Ulmus davidiana* var. *japonica* (Ikeda 1999). *Quadrastichodella nova* Girault, 1922, was originally described from Pentland, Queensland, Australia (Girault 1922). The first description of its biology (as *Flockiella eucalypti* Timberlake) was from California, USA, by Flock (1957). It has since been recorded from various countries in different continents (table 1.1) The presence of *Q. nova* in New Zealand, as indicated in table 1.1, is, however, unconfirmed, since Hawkins & Goeden (1982) quoted Dumbleton (1971) as having recorded it from New Zealand, but that author was in fact referring to another, unidentified *Flockiella* species. Other synonyms for *Q. nova* are *Quadrastichodella obscurata* De Santis, and *Q. eucalypti* (Timb.).

Table 1.1. Recorded distribution and host plants of *Quadrastichodella nova*

Country	Host	Reference
Australia		Girault (1922)
USA (California, Riverside)	*E. umbellata* (Gaertn.) Domin.	Flock (1957); Timberlake (1957)
Argentina (Buenos Aires)		Bouček (1977)
Spain, Italy, Sardinia	*E. resinifera*	Bouček (1977)
Israel		Bouček (1977)
South Africa		Bouček (1977); The Chalcidoidea Specimen Database, ARC-PPRI
New Zealand (possibly inaccurate – see text)		Hawkins & Goeden (1982)
Turkey	*E. camaldulensis*	Doğanlar & Doğanlar (2008)
Republic of the Congo (Pointe Noir)	*E. platyphylla*	The Chalcidoidea Specimen Database, ARC-PPRI

The two descriptions of the biology of *Q. nova* (Flock 1957; Doğanlar & Doğanlar 2008) agreed that the eggs are deposited in the young flowerbuds of eucalypts. Flock (1957) described how a gall "... in one of the four cells of the bud... occupied most of the cell and replaced the ovules ... resembles the seed and is about one and one-half times as large in diameter...". Both Bouček (1988) and La Salle (1994a) incorrectly quoted this description in implying that the entire flowerbud was transformed into one or several galls. In contrast, Doğanlar & Doğanlar (2008) stated that the larvae "...make a gall-like structure by tightly binding 3-4 young seeds...".

According to Flock (1957), seed capsules contained between one and three, but sometimes up to six galls. Galls remained attached to the walls of the capsule, and adults might emerge from the unopened seed capsule or from exposed galls in open capsules. Flock (1957) found no males of this species. He pointed out that, since adults emerged for at least 2 months after removal from the tree, they could easily be transported with seeds to new locations. The validity of this assumption was illustrated by an incident where *Q. nova* (as *Q. eucalypti*) was detected in a Eucalyptus seed lot from Australia during an X-ray examination of quarantine material in India (Gupta *et al.* 2004). Although capsules infested with *Q. nova*

were found all year in California, they were more abundant in October and November than in spring. It is significant that Flock (1957) recorded heavy parasitism of *Q. nova* by "chalcid-flies" during March 1955 and, perhaps for this reason, did not regard *Q. nova* as economically important. The Principal Museum Scientist at the Department of Entomology of the University of California, Riverside, Dr. S. Triapitsyn, has not been able to locate reference material of parasitoids submitted by Flock or Timberlake during 1957 (S. Triapitsyn, University of California, Riverside, pers. comm. 2008), and it is therefore not possible to match them with either of the species associated with *Q. nova* in South Africa.

The first record of *Q. nova* from South Africa was a specimen collected by A. Watsham in Grahamstown (Eastern Cape Province) during December 1973 (Bouček 1977). Thereafter it was recorded from the seed capsules of *E. camaldulensis* collected from Grootkloof (25.51S; 27.24E) (North-West Province) in February 1998; from Brits (25.39S; 27.46E) (North-West Province) in August 2003; on the Klipdrif-Boekenhoutkloof road (25.24S; 28.17E) (Gauteng) in November 2003; and from Colesberg (30°42'25"S; 25°06'34"E) and Hanover (31°13'32"S; 24°15'16"E) (Northern Cape Province), also in November 2003 (The Chalcidoidea Specimen Database of the Biosystematics Division, ARC- Plant Protection Research Institute, Pretoria).

1.2.3 *Leprosa milga* Kim & La Salle, 2008 (Eulophidae: Tetrastichinae)

The genus *Leprosa* was described with *L. milga* as its only species, the type specimen of which originated from Stellenbosch, Western Cape, South Africa (Kim & La Salle 2008) and was submitted as part of this study. The earliest specimens were reared by S. Neser from seed capsules of the *E. camaldulensis* complex collected at Rietondale Research Centre in Pretoria, Gauteng during August 2002, and from seed capsules of the *E. camaldulensis* complex 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).

Kim & La Salle (2008) described *L. milga* as a gall inducer in the seed capsules of "*Eucalyptus* ?*camaldulensis*", based on records from South Africa where it had been the only hymenopteran species to emerge from galled seed capsules. At the time of description, *L. milga* was also known from Italy, where it was reported as causing galls on the seeds of a *Eucalyptus* sp. (Kim & La Salle 2008). Although it still had not been recorded from Australia, Kim and La Salle (2008) regarded it as an Australian species, based on morphological and biological similarities to two endemic Australian genera, *Quadrastichodella* and *Moona*. Since

Q. nova has also been described as a gall inducer in the seed capsules of *Eucalyptus* sp. (Flock 1957) and *M. spermophaga* as a gall inducer in the seeds of *Corymbia* sp. (Myrtaceae) (Kim *et al.* 2005), this would indicate a relationship between the three species.

1.2.4 *Aprostocetus* sp. (Eulophidae: Tetrastichinae)

Aprostocetus Westwood is one of the largest chalcid genera, containing almost 700 described species (La Salle 1994a; Noyes 2003). Together with *Tetrastichus*, *Aprostocetus* is the largest Australian genus in the subfamily Tetrastichinae (Naumann 1991).

Most known species of the genus *Aprostocetus* are internal parasitoids; several species have been described as parasitoids of gall-inducing hymenopterans (Withers *et al.* 2000, La Salle *et al.* 2009b). *Aprostocetus consobrinus* (as *Tetrastichodes consobrinus*) (Girault 1913) and *A. eucalypti* (as *Neomphaloidella eucalypti*) (Girault 1929) were specifically recorded as being associated with galls on eucalypts in Australia. An African species, *A. exertus* La Salle, has recently been described as a parasitoid of the invasive tetrastichine erythrina gall wasp, *Quadrastichus erythrinae* Kim (La Salle *et al.* 2009b), while *A. nitens* and *A. tritus*, two more species from South Africa, were described as parasitoids of another three South African *Quadrastichus* species (Prinsloo & Kelly 2009). *Aprostocetus* species have been recorded as parasitoids within galls caused by psyllids, or in the eggs of orthopterans (La Salle 1994b). As mentioned earlier, one example of an unidentified *Aprostocetus* sp. feeding as an ectophagous larval predator on the adult stage of a gall-forming nematode, *Subanguina mobilis* (Anguinidae) is also known (Van den Berg *et al.* 1990).

Gall-inducing *Aprostocetus* species have also been described, e.g. an unidentified species that develops within, and is believed to be the originator of, galls on the midribs of the leaves of lemon gum, *Corymbia citriodora* (as *Eucalyptus citriodora*) (Beardsley & Perreira 2000; La Salle 2005).

An undescribed *Aprostocetus* sp. (O.C. Neser, ARC-PPRI, pers. comm., February 2007) forms part of the complex of chalcidoid species associated with galls in the seed capsules of *Eucalyptus camaldulensis* in South Africa. The Chalcidoidea Specimen Database of the Biosystematics Division, ARC- Plant Protection Research Institute, Pretoria has the following specimens on record, all of which were recorded as part of the current investigation: HYMC03651 – Stellenbosch (33°56'45"S; 18°51'21"E); HYMC04805 – Citrusdal (32°32'44"S; 19°00'32"E); HYMC04806 – Citrusdal (32°32'44"S; 19°00'32"E); HYMC04807 – Citrusdal

(32°32'44"S; 19°00'32"E); HYMC04808 – Wolfdrif (32°03'01"S, 19°04'00"E); HYMC04829 – Citrusdal 15km N (32°25'22"S; 18°57'34"E); HYMC04830 – Rietondale (25°43'39"S; 28°14'13"E); HYMC04831 – Rietondale (25°43'39"S; 28°14'13"E); HYMC04832 – Wolfdrif (32°03'01"S, 19°04'00"E); HYMC04833 – University of Pretoria Experiment Farm (25°45'11.09"S; 28°15'11.02"E); HYMC04834 – Rietondale (25°43'39"S; 28°14'13"E); HYMC04835 – Nylstroom (24°40'23"S; 28°30'15"E).

1.2.5 Eulophid #4 (Eulophidae: Tetrastichinae, genus indet.)

This species, belonging to an as yet undescribed genus (O.C. Neser, ARC-PPRI, pers. comm. 2007), forms part of the complex of chalcidoid species associated with galls in the seed capsules of *Eucalyptus camaldulensis* in South Africa and is characterised by a distinct elongate hypopygium. The Chalcidoidea Specimen Database of the Biosystematics Division, ARC- Plant Protection Research Institute, Pretoria has the following specimens on record: HYMC02999 – Grootkloof (25.51S; 27.24E) [collected by S. Neser]; HYMC04809 – Citrusdal (32°32'44"S; 19°00'32"E); HYMC04810 – Citrusdal (32°32'44"S; 19°00'32"E); HYMC04811 – Wolfdrif (32°03'01"S, 19°04'00"E); HYMC04812 – Wolfdrif (32°03'01"S, 19°04'00"E); HYMC04813 – Wolfdrif (32°03'01"S, 19°04'00"E); HYMC04814 – Waterval-Onder (25°26'51"S; 30°57'59"E); HYMC04815 – Citrusdal 15km N (32°25'22"S; 18°57'34"E); HYMC04816 – Welverdiend (32°04'52"S; 18°49'38"E).

1.3 GALLS AND GALL-INHABITING INSECTS

1.3.1 Galls and gall development

A gall (cecidium) can be described as an atypical plant growth, the result of a highly specific and specialized reaction by a plant to the presence of, and wounding and feeding by, a gall inducer (Mani 1992; Dreger-Jauffret & Shorthouse 1992; Rohfritsch 1992). The development of a gall depends on the continued presence and feeding activity of the larva of the gall inducer (Shorthouse & Lalonde 1988). The physiological changes that induce gall formation are produced by the plant cells, not by the cecidozoa (gall inducers) (Mani 1992). Nevertheless, galls are unique to the species of gall inducer (Dreger-Jauffret & Shorthouse 1992), representing the extended phenotypes of the gall-inducer's genes (Dawkins 1982; Stone & Schönrogge 2003).

By drawing photoassimilates from other parts of the plant, galls act as physiological sinks. As a reaction to wounding and feeding by the cecidozoa, the plant cells proliferate and

synthesize starch and stress metabolites, which provide the cecidozoa with shelter and food (Rohfritsch 1992).

Viruses, bacteria, fungi, nematodes, mites and insects have been recorded as gall inducers (Mani 1992). The insect order Hymenoptera contains the most highly evolved gall inducers, which cause the most complex and highly evolved of all galls (Dreger-Jauffret & Shorthouse 1992). Hymenopteran gall inducers are found in both suborders Symphyta (family Tenthredinidae) and Apocrita. Within the latter, gall inducers occur in two superfamilies: Chalcidoidea (mainly the families Pteromalidae, Eurytomidae, Agaonidae, Eulophidae and Tanaostigmatidae) (La Salle 2005) and Cynipoidea (family Cynipidae) (Dreger-Jauffret & Shorthouse 1992).

Most of the galls induced by Chalcidoidea are situated in buds or growing points of shoot apices, although some are also found on leaves, roots and flowers. Chalcidoid-induced galls are typically solid, hard and woody with numerous superficially isolated larval chambers in the cortex (Dreger-Jauffret & Shorthouse 1992), although gall morphology may not always be an indication of the taxonomy of the gall inducer. The eggs are usually deposited in a row. The swelling of the attacked organ appears opposite the oviposition scar, and causes the tip of the shoot to bend horizontally or allows gall tissue to protrude out of the shoot or leaf vein (Rohfritsch 1992).

Galls develop through four basic stages: initiation, growth and differentiation, maturation, and dehiscence, as described by Rohfritsch (1992) and Brooks & Shorthouse (1998). Based on the mechanisms whereby galls are initiated and whereby their growth is controlled, three basic types of galls can be recognized: the cecidomyid, tenthredinid and cynipid types (Rohfritsch 1992). Most hymenopteran galls are represented by the latter type (Rohfritsch 1992).

Hymenopteran galls are initiated by the act of oviposition by the adult. In cynipid galls, specifically, several factors are involved in the initiation process: the wounding of plant tissues by the ovipositor; the application of ovipositional fluids; the proteolytic, cellulolytic and pectinolytic activity of the egg (which is the most important component in gall initiation); and the activity of the newly hatched larva (Rohfritsch 1992; Brooks & Shorthouse 1998). The initiation process overrides the normal growth pattern of a group of plant cells at the oviposition site and modifies them physiologically. The plant cells around

the egg de-differentiate, they become re-activated to proteosynthesis and RNA synthesis, undergo hypertrophy and hyperplasia to produce a pad of tissue supporting the egg; the cells beneath the egg then lyse and collapse to form a larval cavity, while new cells are produced around the cavity. Upon hatching, the larva moves into this cavity and becomes surrounded by young growing tissue (Rohfritsch 1992). Brooks & Shorthouse (1989) noted that the chalcid egg, however, lacked the lytic action of cynipid eggs. Whereas it is mainly the larval stage that influences gall growth and tissue differentiation in galls of the cynipid type, in galls induced by chalcids the maternal influence on gall morphology is more important (Rohfritsch 1992).

The newly formed cells now increase their biomass vastly by cell division and enlargement, at a rate depending on the feeding activity of the larva. The larva feeds by applying salivary fluids to the plant cells to liquefy their contents, and then sucks up the liquids. The nutritive cells closest to the larva - which are most highly stimulated by the feeding activity, the presence of salivary enzymes and the release of various plant cell substances into the larval cavity - grow largest and produce large quantities of proteins. The plant cells further away proliferate to create nutritive tissue along the inner surface of the larval chamber. Vascular tissue then develops amongst the growing cells to join the normal vascular tissue of the attacked organ (Rohfritsch 1992; Brooks & Shorthouse 1998). The continual presence of the feeding larva is necessary for the formation and maintenance of nutritive tissue; if the larva dies, callus tissue will be formed instead (Shorthouse & Lalonde 1988).

The gall matures when the insect reaches its last larval instar and consumes all the nutritive tissue that has accumulated around it. In many cases, a sclerenchyma sheath develops around the layers of nutritive tissue, near the vascular tissue, separating the gall into an inner and an outer section. Towards the end of the gall's maturation phase, the flow of sap to the gall stops, the gall ceases to be a nutrient sink, an abscission layer may separate the inner from the outer gall, and the inner gall may fall to the ground to allow the gall inhabitants to escape. However, chalcid gallmakers overwinter and pupate within the gall and escape through exit holes. By the time the insect has completed its pupal stage, nothing is left of the nutritive zone but a lining consisting of a few partially eaten cells and the remnants of cell walls bordering the sclerenchyma sheath (Rohfritsch 1992; Brooks & Shorthouse 1998).

Evidence exists to indicate that gall induction has arisen many times in the Chalcidoidea, having evolved independently along at least three pathways: from parasitoids, from seed or ovule feeders, and from stem borers (La Salle 2005). Evidence supporting the theory that phytophagous forms (gall-inducers or inquilines) among the chalcidoids evolved from parasitoids is provided by the occurrence of entomophytophagy, which refers to the phenomenon that many extant parasitic Torymidae and Eurytomidae attack immature host larvae (Askew 1975) and, lacking the ability to delay their larval development until the host larva is mature, sometimes continue feeding on gall tissue after having consumed the host larva. According to the theory, during the next stage in the evolution the ovipositing female then becomes independent of the host larva, resulting in a completely phytophagous gall-inducing or inquiline) habit (Roskam 1992).

1.3.2 Gall communities

Galls provide a favourable habitat for the development of microcommunities, due to the high concentrations of very nutritious substances within gall tissues, the vulnerability of the encapsulated, sedentary gall-inducer, and the presence of cells with near-meristematic conditions (Mani 1992, Roskam 1992). Complex galls might contain predators, parasitoids, inquilines, and successors. Frequently, the composition and interrelationships of microcommunities are characteristic of the species of gall inducer and its gall (Mani 1992).

- *Parasitoids:* During its larval stage, a parasitoid develops either internally or externally upon a single host individual, which eventually dies as a result of the attack. The adults of most parasitoids are free-living, and feed on nectar or body fluids (Clausen 1962; Dixon 2000; Gordh & Headrick 2001). In the older literature, the term "parasite" was used for such insects (Clausen 1962).

  Two deviations from the normal biology of parasitoids have been recorded in gall communities. Both of them occur when the parasitoid larvae hatch before the insect hosts are large enough to allow complete development of the parasitoid. In the first case, the larva of the parasitoid feeds initially on plant tissue, until the larva of the host can support its development (Stone, G.N. & Schönrogge, K. undated. Oak gall communities webpage http://www.homepages.ed.ac.uk/amegilla/Oak%20 gall%20communities, accessed 06/12/2007). In the second case, the parasitoid larva starts its development by consuming the insect host, and then completes its development by consuming the plant tissue within which the insect host lived – known

as entomophytophagy (Roskam 1992, La Salle 2005). Both of these methods provide parasitoid larvae with an additional food source and allow the adult parasitoids to oviposit before the gall wall becomes too tough or thick to penetrate (Stone & Schönrogge 2003).

- *Predators:* A predator, according to Clausen (1962), is free-living during its larval stage; it attacks and kills the host outright and requires a number of individuals to provide sufficient food to bring it to maturity. The predator is larger than the prey, and the food sources of the adults and immature stages are often the same. An example of a eurytomid larva developing as a predator of a seed-galling eulophid larva, and which is able to move to neighboring galls by chewing holes in order to attack eulophids in other galls, was described by Gibernau *et al.* (2002).

- *Inquilines:* Inquilines are defined as organisms that occupy galls induced by another insect, that use the gall tissue, but do not feed directly on the gall inducer or other insects, although the larva of the gall inducer might be killed by the female inquiline during oviposition, or indirectly by modification of the gall (Brooks & Shorthouse 1998; Ferraz & Monteiro 2003; Stone, G.N. & Schönrogge, K. undated. Oak gall communities webpage http://www.homepages.ed.ac.uk/amegilla/Oak%20gall%20communities, accessed 06/12/2007). Malyshev (1968) used the term "inquiline" in a wider sense, including hymenopterans that deposit their eggs in an insect gall, and whose larvae attack the eggs or larvae of the gall-inducer and suck out their body fluids, whereafter they continue to feed on the walls of the gall. In this dissertation, the term "entomophytophagous parasitoid" will be used for the latter type of life history, and the term "inquiline" will be reserved for insects using the gall of another insect species without feeding on the larvae of the gall-inducer. Some inquilines do not affect the gall inducer, provided there is sufficient nutritional tissue. However, lethal inquilines feed and develop faster than the gall inducer, thus causing the gall inducer to starve to death, or causing the death of the gall inducer by interfering with the opening mechanism of the gall (Mani 1964). Many lethal inquilines modify the gall morphology, e.g. by inducing tissues and chambers of their own inside the host gall ("endogalls", as described by Ferraz & Monteiro (2003)), and cause the death of the gall inducer larva by gradually reducing the space available to it (Brooks & Shorthouse 1998; Ferraz & Monteiro 2003; Medianero *et al.* 2007; van Noort *et al.* 2007).

- *Successors:* Successors are insects that use empty galls as their shelter once the original inhabitants have emerged (Tscharnke 1999; Yukawa *et al.* 2005).

The information summarised in this chapter was used to facilitate the correct interpretation of observations that were made when dissecting flowerbuds, flowers and seed capsules of the *E. camaldulensis* complex, containing immature hymenopterans in galls, during the main part of this study (chapter 3). The juveniles of hymenopterans are extremely small, and it is not possible to identify them to species level during their immature stages, but the numerous pieces of information from the literature could serve as clues to the identity and biology of the juveniles encountered during the study, until the tentative identifications could be tested by using molecular techniques (chapter 3). The validity of assumptions made in the literature descriptions of three of the members of the gall community (*M. zebrinus*, *Q. nova* and *L. milga*) could also be tested against observations made during this study.

CHAPTER 2: GEOGRAPHICAL DISTRIBUTION AND EMERGENCE PATTERNS OF THE GALL INHABITANTS, CORRELATED WITH THE PHENOLOGY OF THE HOST TREE

Of the hymenopteran species that were known to be associated with galls in the seed capsules of *E. camaldulensis* in South Africa prior to the initiation of this study, *Megastigmus zebrinus* was first discovered during 1998, in the Western Cape, and the first records of *Quadrastichodella nova* for South Africa were from the Eastern Cape in 1973 and from the North West Province, during 1998. When research into the biological control of invasive eucalypts was first contemplated, the question arose as to how commonly these two species occurred in South Africa. A nation-wide survey was therefore undertaken, the results of which are reported here.

PART I: GEOGRAPHICAL DISTRIBUTION

2.1 METHODS (Tables 2.1 & 2.2)

Between 2003 and 2008, surveys of trees belonging to the *Eucalyptus camaldulensis* complex in South Africa were undertaken by the author, together with S. Neser and A.B.R. Witt (both ARC-PPRI at that time). The variability of *E. camaldulensis* in South Africa, coupled with its ability to hybridize with the closely related *E. tereticornis* Sm. (forest red gum) (Venkatesh & Thapliyal 1993), made it difficult to identify a tree specimen with certainty as *E. camaldulensis* in South Africa. Therefore, specimens were selected for study if they had more characteristics of *E. camaldulensis* than of *E. tereticornis*, and will be referred to as belonging to the "*E. camaldulensis* complex".

The Gauteng, Mpumalanga, North West, Limpopo, Free State and Northern and Western Cape Provinces were sampled. Although *E. camaldulensis* also occurs in KwaZulu-Natal and the Eastern Cape Province, these two provinces were not included in the survey. Wherever possible, localities were revisited repeatedly and sampled throughout the year, to ensure that insects emerging during different seasons were detected.

On each sampling occcasion, several branches of one tree in the *E. camaldulensis* complex, containing approximately 250 ml to 500 ml of seed capsules, were collected and taken to the laboratory. All leaves, flowerbuds, flowers and young, soft capsules were discarded, and only the fully-developed, hardened seed capsules (either green or brown) on a bare section of stem were placed into carton emergence boxes which were kept in a room at the Rietondale Weeds Laboratory under ambient temperatures and light conditions.

Insects that emerged from each sample were recorded. The results (table 2.1) were analyzed to indicate:

- sample sites per province;
- number and date of sample collections;
- chalcidoid species that emerged per site.

Other hymenopterans that emerged only rarely were listed in table 2.2.

Voucher specimens of all species that emerged during this study were deposited in the National Collection of Insects, Pretoria.

2.2 RESULTS (Table 2.1)

Of the 60 sites sampled during the five-year period between 2003 and 2008, seed capsules from 46 collecting sites yielded specimens of one or more of the following five hymenopteran species: *Megastigmus zebrinus*, *Quadrastichodella nova*, *Leprosa milga*, *Aprostocetus* sp. and an undescribed species, referred to as Eulophid #4, in an apparently undescribed tetrastichine genus. The results of the surveys are summarised in table 2.1.

At only 12 of the 26 sites from which *M. zebrinus* was reared, did *M. zebrinus* emerge as the only hymenopteran species - in concurrence with the report by Grissell (2006). However, all of these sites had only been sampled once. Sites that were sampled repeatedly were more likely to yield three or more species of hymenopterans than those that were sampled once only. This indicates that at least some of the hymenopterans were only around at certain times of the year, and serves to illustrate the importance of monitoring a site repeatedly, at different times of year, to ensure that all members of a suite of gall inhabitants are captured.

Table 2.1. 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.; #4 = Eulophid #4.

Province	Locality	Sample date(s)	# samp-les	Wasp species				
				Mz	*Qn*	*Lm*	*Ap*	*#4*
Gauteng	Rietondale tree 2, Pretoria 25°43'39"S; 28°14'13"E	Mar 06; Aug 07	4	x		x		
	Rietondale tree 3, Pretoria 25°43'39"S; 28°14'13"E	Dec 04	2	x	x			
	Lynnwood Ridge, Pretoria 25.46S; 28.17E	Dec 2003	1	x				
	Bronkhorstspruit 25°53'37"S; 28°40'59"E	Jan 2006	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					
Mpuma-langa	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					
	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			

Province	Locality	Sample date(s)	# samples	Wasp species				
				Mz	*Qn*	*Lm*	*Ap*	*#4*
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		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						
	Citrusdal 15 km S 32°25'22"S; 18°57'34"E	Dec 05; Jan 07; Oct 07	3	x	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 32°35'33"S; 19°00'39"E	Oct 07	1	x	x	x	x	
	Tulbagh /Worcester 33°29'15"S; 19°11'48"E	Dec 05						
	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		
Totals	**60**			**34**	**29**	**17**	**3**	**2**

It was now clear that the annual emergence pattern of the members of the suite of gall inhabitants needed to be examined more closely. Due to the intimate association of gall inhabitants with their host plant, insect emergence needed to be correlated with the phenology of the reproductive structures on the trees. That aspect of the study forms the second part of this chapter.

Table 2.2. Additional hymenopteran species emerging rarely 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)

PART II: EMERGENCE PATTERN OF THE GALL INHABITANTS, CORRELATED WITH THE PHENOLOGY OF THE HOST PLANT

The life cycles of gall-forming hymenopterans are closely synchronised with the phenology of their host plants (Yukawa 2000; Imai & Ohsaki 2006). After emergence, adults of a gall inducer may have only a short opportunity in which to mate and oviposit, especially those that do not take in food as adults, and therefore have a lifespan of only a few days (Yukawa 2000). To induce galls, the insects require physiologically active plant tissue, which may only exist during a limited stage of plant development, thus forming a "phenological window" for gall induction (Imai & Ohsaki 2006). In parasitoids, oviposition time might be limited to the period when the gall wall is thinnest (when the larva of the gall-inducer has consumed most of the nutritive tissue). If, as in this study, the gall occurs inside the fruit, oviposition might also be restricted to the period before the fruit integument becomes woody. In addition, a parasitoid can only obtain sufficient nutrients to complete its development once its prey larva has reached a certain minimum size, which requires synchronization between the parasitoid and the gall inducer. The growth pattern of gall inducers might therefore limit parasitism by narrowing its window of vulnerability to parasitoids (Ito & Hijii 2002). Instances have been reported where host specificity in gall-inducing insects is driven by the synchrony of oviposition and flower availability (Vitou *et al.* 2008). Yukawa (2000) cautioned that, without phenological information, various field data would be incorrectly evaluated.

The results presented above indicate that adults of at least some of the different hymenopteran species that are associated with seed capsules from the same *E. camaldulensis* tree emerge only at particular times during the year. To investigate these seasonal emergence patterns, a detailed study of selected *E. camaldulensis* trees, spanning a year, monitored the emergence of all the hymenopteran species associated with the seed capsules, from samples collected at regular intervals throughout the year. Information on the emergence patterns was needed to ensure that the different developmental stages of each species could be followed reliably during more-intensive studies of the insects. Simultaneously, the phenology of the reproductive structures of *E. camaldulensis* was monitored because this could explain the development patterns of the gallformer. Apart from the fact that *E. camaldulensis* flowers in summer (Poynton 1979), no further information was available about the phenology of this species at the time.

2.3 METHODS

Three experimental trees in the *E. camaldulensis* complex were selected to study the emergence of gall inhabitants and the phenology of the host trees, based on the following criteria:

- Although the ideal would have been to monitor trees from different climatic regions of South Africa, it was not practicable to monitor geographically distant sites at fortnightly intervals. It was therefore decided to restrict the trees to within a radius of 20 km from Rietondale Research Station in Pretoria, to accommodate affordable travelling time and cost. This regrettably excluded any trees growing along watercourses.
- Emergence holes had to be present in some of the dry seed capsules on the ground under the tree, which was regarded as an indication that the gallformer was present.
- The environment of the tree had to be safe enough for an unaccompanied person to work in, and general access to the tree had to be restricted, so as to safeguard the tags and sleeves and the tree itself against vandalism and other forms of interference.
- The lower branches had to be accessible from the ground, or by using a readily-portable step ladder.

Based on these criteria, the following three trees were chosen:

- Rietondale (25°43'50.97"S; 28°14'30.64"E): A very large tree, approximately 23 m high and 20 m canopy diameter, growing as part of a group of eucalypts in disturbed grassland on the experiment farm at Rietondale, Pretoria. The entire experiment farm is

fenced in, and guarded by a security company. This tree receives no water other than rain, and has shown signs of drought stress during the dry winter months. The tree died early in 2008, probably from infestation by an Australian hemipteran, *Thaumastocoris peregrinus* Kirkaldy (Heteroptera: Thaumastocoridae: Thaumastocorinae) (Jacobs & Neser 2005) and the eucalypt shoot psyllid, *Blastopsylla occidentalis* Taylor (Halbert *et al.* 2001).

- University of Pretoria experiment farm (25°45'11.09"S; 28°15'11.02"E): A very large, spreading tree, approximately 23 m high and 24 m canopy diameter, growing as part of a small group of eucalypts behind the dairy section, and close to a number of staff residences, in a park-like open area. The staff gardens and lawns are regularly irrigated, and the test tree possibly received some additional ground water from this source. Access to the experiment farm is limited to students, staff and card holders.
- Rooihuiskraal North, in a security complex, Craddock Park (25°52'41.39"S; 28° 8'25.20" E). A large tree, approximately 25 m high and 20 m canopy diameter, growing singly on the communal lawn of the complex, which is regularly irrigated and receives run-off water from the washing of residents' cars. Access is limited to residents, visitors and staff with security clearance, but at several occasions tags were removed by residents' children or lost due to pruning by the gardening staff.

2.3.1. Monitoring the emergence of hymenopterans from the seed capsules

At fortnightly intervals, from the first or second week of April, 2007, until the end of March, 2008, approximately 250-500 ml of fully developed seed capsules (either green, or brown and woody) were collected from each of the selected test trees described above, and placed in an emergence box to collect hymenopteran adults from the galls. Fully developed seed capsules were known to be the only stages in which the immature hymenopterans were able to complete their development in an emergence box. The emergence boxes were monitored until December 2008 and all insects that emerged were recorded per insect species and date of emergence, as well as noting from which sample (emergence box) each emerged. Thereafter the emergence boxes were emptied and the number of seed capsules per sample recorded. The capsules of each sample were retained for future reference.

For each of the three test trees, the emergence records were analysed in two ways to determine, firstly, which species emerged from each sample (to indicate whether the species composition within samples changed throughout the year) and, secondly, when each species emerged (to indicate the temporal emergence patterns of the five species).

2.3.1.1 The species composition of the gall inhabitants that were reared from each sample (Fig. 2.1): Emergence of adults was recorded against sample date in order to show which hymenopteran species emerged from a particular month's samples. This was done to ascertain whether the observation of Grissell (2006), where only *M. zebrinus* emerged from a sample of seed capsules collected from *E. camaldulensis* in the Western Cape, South Africa, applied here. This was accomplished by examining the results from samples collected during each month separately, and noting how many individuals of the various chalcidoid species emerged until December 2008 from each monthly sample. The number of each of the five hymenopteran species that emerged from a particular sample of capsules was then plotted against the month during which the sample had been collected. The number of hymenopterans was expressed as individuals per 100 seed capsules, and a 10-base logarithmic scale was used, to accommodate the large range in emergence numbers (fig. 2.1).

2.3.1.2 The emergence pattern of each of the five hymenopteran species from the combined samples from the particular test tree (Fig. 2.2; Table 2.2): This analysis determined when each of the insect species emerged from the seed capsules. Only the emergence date was used while the collection date of the sample from which the insect emerged, was disregarded. All the samples for each month in which there was emergence were combined to show the monthly number of emergences per hymenopteran species. The number of hymenopterans was expressed as individuals per 100 seed capsules, and a 10-base logarithmic scale was used, to accommodate the large range in emergence numbers (fig. 2.2). The total numbers of each hymenopteran species that emerged from the combined samples for the year were tabulated per test tree (expressed as emergences per 100 capsules) (table 2.2).

A preliminary examination of the results indicated obvious differences in emergence patterns between samples from the three test trees. Although a statistical comparison of replicates (the three test trees that were sampled) is regarded as inappropriate, the potential relevance of the differences between the samples made it nevertheless desirable to investigate this aspect. The Kruskal-Wallis test was chosen for this purpose, being a non-parametric independent group comparison, which does not assume normally distributed samples or equality of variance, and can cope with the small samples, which are often unavoidable in the field-collected data.

The three sampling trees were therefore compared with regard to the relative emergence numbers of the various chalcidoid species from samples from each, using the Kruskal-Wallis test. The emergence of various chalcidoid species was also compared per season, using the same test; for this purpose, emergences from samples taken from all three test trees had to be combined.

2.3.1 **Monitoring of tree phenology** (Fig. 2.3)

The general appearance and morphology of the reproductive structures of eucalypts are very distinctive and atypical. Therefore the following description, based upon Brooker *et al.* (2002), is presented in order to describe the morphology of buds, flowers and fruit of eucalypts and to define the terminology that will appear later in this chapter:

> "In most eucalypt species, including *E. camaldulensis*, the buds occur in clusters of odd numbers (often 3 or 7) (fig. 2.3 B) on single stalks in the axils of the leaves. The very young inflorescence is held within deciduous bracts, which are soon shed. The flowers are small and whitish, the petals being fused to form an inner operculum (fig. 2.3 D), which sheds just before flowering when the stamens expand and are almost ready to shed their pollen. The sepals are also fused to form an outer operculum, which sheds early in bud development, leaving a scar (fig. 2.3 B) around the middle of the bud. Within the base of the buds is the ovary, sunk into the expanded top of the pedicel (individual bud stalk), known as the hypanthium (fig. 2.3.C). It contains vertical rows of ovules. The top of the ovary is surmounted by the style which terminates in the stigma. Pollination takes place by wind, insects, small birds or mammals. The fertilized ovules at the base of the placentae mature into the seeds, while the ovular structures on the upper part of the placenta are infertile or unfertilized and give rise to sterile particles smaller than seeds known as the chaff.
>
> Following fertilization, the stamens fall from the flower, the style is shed, and the remaining structure becomes woody and matures into a fruit (fig. 2.3 E-G). The rim of the fruit comprises the scar or circular "platform" where the operculum was attached, then on the inner side, the narrow or broad ring of tissue that bore the stamens, and finally a band of tissue, known as the disc, which links the rim with

the ovary roof (fig. 2.3 F). In *E. camaldulensis*, the disc is raised and ascends to an uplifted roof.

The roof of the ovary breaks into valves (fig. 2.3 G) which spread and allow the seed to shed. In the red gums, including *E. camaldulensis*, the ovary splits into 3 or 4 valves which are usually strongly exserted. Until the vascular connections between the individual fruits held in the crown and the parent tree are broken, the valves will not open. Otherwise, eucalypt fruit are held on the branchlets often for years. Seed and chaff particles from detached fruits are released within 24 hours if allowed to dry out. The seeds of *E. camaldulensis* are cuboid in shape."

For the purpose of recording the phenology of the reproductive structures of trees in the *E. camaldulensis* complex, these structures were divided into distinct developmental stages. The stages had to be externally discernible, to ensure that they could be identified during each sampling event without damaging them. It was also necessary to record the internal morphology of each of the externally discernible stages, because that was regarded as the most likely aspect to which the gall-inhabitants would be adapted. Based on these requirements, seven stages (stages A to G) were identified, from the youngest flowerbuds until the old, woody seed capsules. Photos were taken of the external and internal view of each of the stages, and the internal morphology of each stage was matched with the external appearance (fig. 2.3).

During the first visit to each of the three test trees described above, ten clusters of flowerbuds, flowers or capsules belonging to each of the seven developmental stages were marked on each tree by means of white carton price-tags with a piece of string attached. Frequently the individual structures in a cluster would belong to different developmental stages; in such cases, the stage of only the oldest individual structure in a cluster would be recorded. On each of the three test trees there would thus be 70 tagged clusters, ten each belonging to each of the stages A to G. If a certain stage was absent during the first visit, tags would be attached as soon as the particular stage appeared.

Every second week thereafter, until the last week in March, 2008, each of the test trees was re-visited, and the following aspects were recorded for each:

2.3.2.1 Development of tagged clusters of reproductive structures (Fig. 2.4): The developmental stage reached by the oldest individual in each of the tagged clusters was recorded for each of the test trees. At the end of the trial period of a year, the average duration of each of the identified developmental stages was calculated for each of the three test trees individually, expressed as number of weeks. For this purpose, all the individual reproductive structures of a specific stage, for which both the beginning and end dates in that stage were known, were included in the calculation. If either a tagged cluster or the tag itself disappeared during the course of the experiment, the length of the last, incomplete developmental stage was disregarded for that cluster. Similarly, the duration of the stage during which a particular cluster was tagged, was also disregarded during the calculations, owing to the uncertainty as to how long the cluster had been in that developmental stage before it was tagged. The exceptions were stage A (representing the very young flowerbuds), which was included nevertheless, although it was not possible to determine the age of each individual at the time of tagging with certainty, and stage G (representing the oldest, woody seed capsules), of which the same was true, but in addition most of them had not yet fallen to the ground, and therefore had not reached the end of the stage, by the time that the trial was concluded. The total duration of each development stage, calculated for each of the three test trees, was plotted on a bar chart (fig. 2.4).

2.3.2.2 Relative abundance of development stages of the tree at different times of the year (Fig. 2.5): During each sampling event, a visual estimate was made of the relative abundance of each of the seven development stages of *E. camaldulensis*, expressed as a percentage of the total number of reproductive structures on the tree at that particular time. The average abundance per calendar month was calculated for each of the developmental stages. These results were plotted on an area graph, where the contribution of each stage during a particular month was represented by an area, with the areas of all the different stages for a specific month stacked to represent a total of 100 %. The results for each of the three test trees were plotted separately (fig. 2.5).

2.4 RESULTS

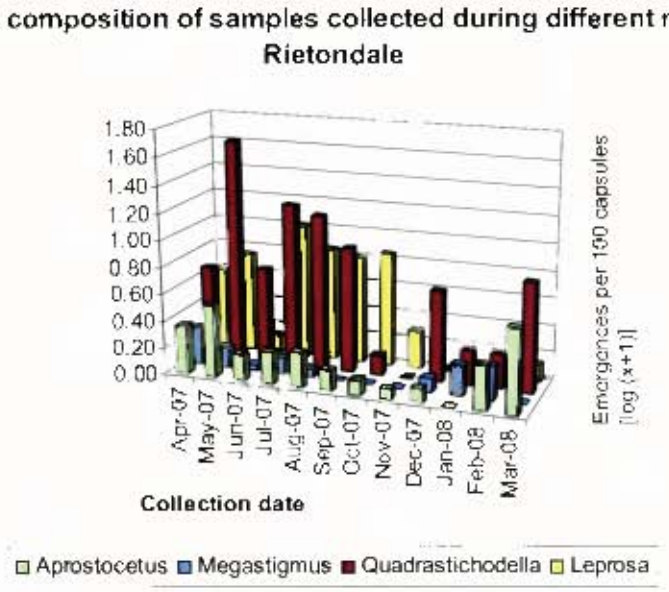
2.4.1 Emergence of hymenopterans from seed capsules

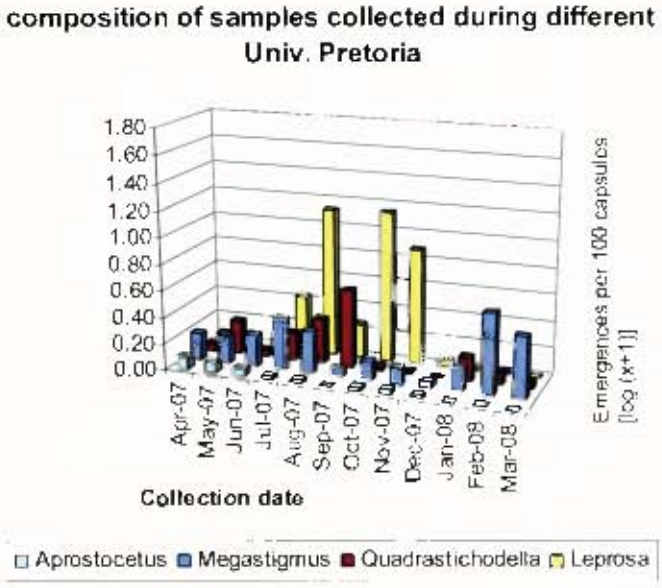
2.4.1.1 Species composition of chalcidoids emerging from capsule samples collected at different times of the year (Fig. 2.1): The relationship between the collection date of a capsule sample and the species composition of the chalcidoids that emerged from the sample varied greatly between the Rietondale test tree on one hand, and the University of Pretoria and the Rooihuiskraal test trees on the other.

Almost all samples taken from the Rietondale tree yielded adults of *Q. nova, L. milga, M. zebrinus* and *Aprostocetus* sp. The exceptions were: the samples collected during December 2007-February 2008, from which *L. milga* was absent; those collected during November 2007, from which no *Q. nova* emerged; and those collected during January 2008, which yielded no *Aprostocetus* sp. In contrast, *M. zebrinus* only emerged from samples collected from April to August 2007, and again from December 2007 to January 2008, although the numbers were lower than those of the other three species.

Samples collected from the other two trees differed from samples from Rietondale in several respects: with a few exceptions, *L. milga* emerged only from samples taken between June and November 2007, and *Q. nova* was reared from Rooihuiskraal samples collected almost exclusively from May to November 2007, and from University of Pretoria samples taken during all months except September to December 2007. *Megastigmus zebrinus* was reared from all University of Pretoria samples, and from all Rooihuiskraal samples except April and December 2007. The numbers of *Aprostocetus* sp. that emerged from these two test trees were too low to reveal any pattern of emergence.

To summarize, there was no month, between April 2007 and March 2008, during which a sample of seed capsules from any of the three test trees yielded adults of *M. zebrinus* only (i.e. there were always also adults of one or more of the other hymenopteran species). Therefore the results of this study do not support the observation of Grissell (2006), who reported that the sample of seed capsules he collected in the Western Cape yielded only *M. zebrinus.*





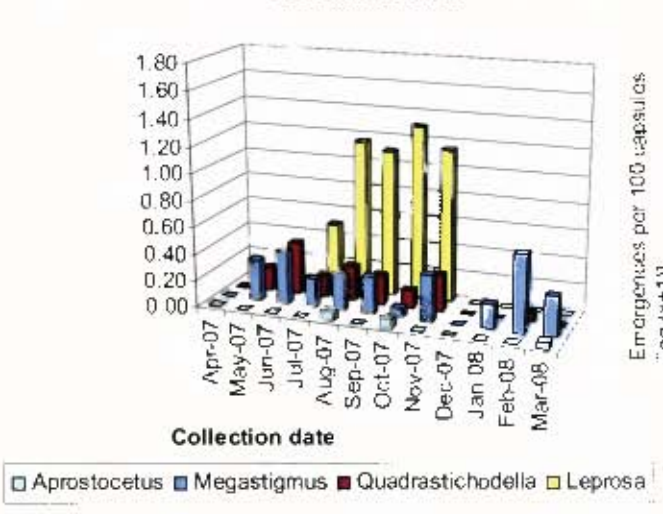


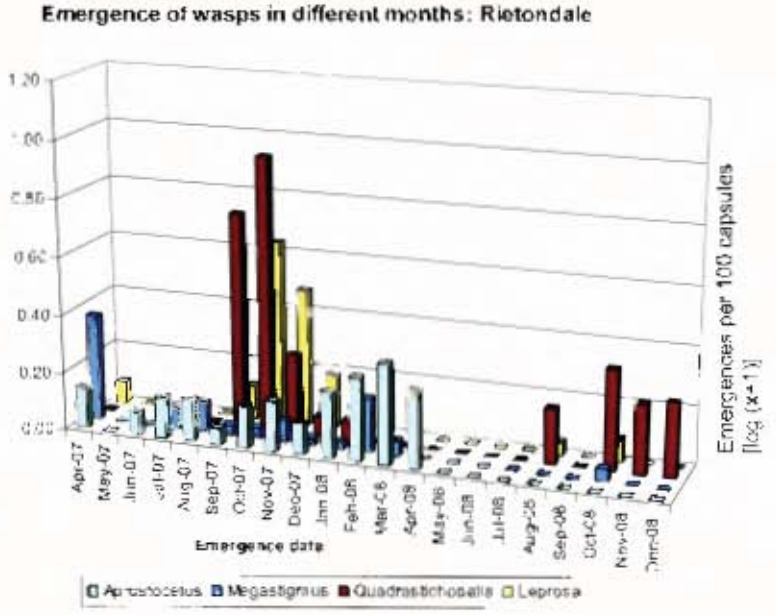
Figure 2.1. Species composition of gall inhabitants in samples of seed capsules of *Eucalyptus camaldulensis* from different sites near Pretoria, collected on different dates

2.4.1.2 Emergence patterns of the various hymenopteran species (Fig 2.2; Table 2.3): The emergence numbers of the following four chalcidoid species differed significantly between seasons, when combining the samples from the three test trees: *M. zebrinus* (H= 16,4; df = 4,54; P = 0,0025), *Q. nova* (H = 22,39; df = 4,54; P = 0,0002), *L. milga* (H = 22,54; ; df = 4,54; P = 0,0002) and *Aprostocetus* sp. (H = 9,83; ; df = 4,54; P = 0,0434).

In the discussion of the emergence pattern of hymenopteran species, two aspects will be considered: a) the period of emergence, and b) the numbers of individuals that emerged.

Regarding the emergence periods, fig. 2.2 illustrates the emergence patterns of each of the chalcidoid species. *Quadrastichodella nova* had an emergence peak during early summer, and emerged from August/September 2007 until December 2007/January 2008, and again from October to December 2008, although the second emergence period started already during August 2008 in samples from the Rietondale tree. *Leprosa milga* had a similar emergence peak, except that it was a bit later: it emerged from September/October 2007 until January/April 2008, and had a second, smaller peak from August to October 2008 but from samples from only two of the test trees (Rietondale and University of Pretoria). There was also a small, early emergence of *L. milga* in samples from the Rietondale tree during April 2007. *Megastigmus zebrinus* emerged during April 2007 from samples taken from only the Rietondale tree, thereafter from all samples from July 2007 to March 2008, and again in October 2008; from samples from only the University of Pretoria tree some individuals emerged during December 2008. The emergence period of *Aprostocetus* sp. varied most between sample sites: From samples taken from Rietondale it emerged during every month from April 2007 until April 2008, with the exception of May 2007; from samples from the other two trees it emerged only occasionally, between June and December 2007 (from University of Pretoria samples) and between November 2007 and March 2008 (from Rooihuiskraal samples). No more specimens of *Aprostocetus* sp. emerged a month or longer after the last samples had been collected. Eulophid #4 was reared only from samples taken from the University of Pretoria tree, where one specimen each emerged during July, September and December. For this reason, this species was not included in the histogram (fig. 2.2).

A



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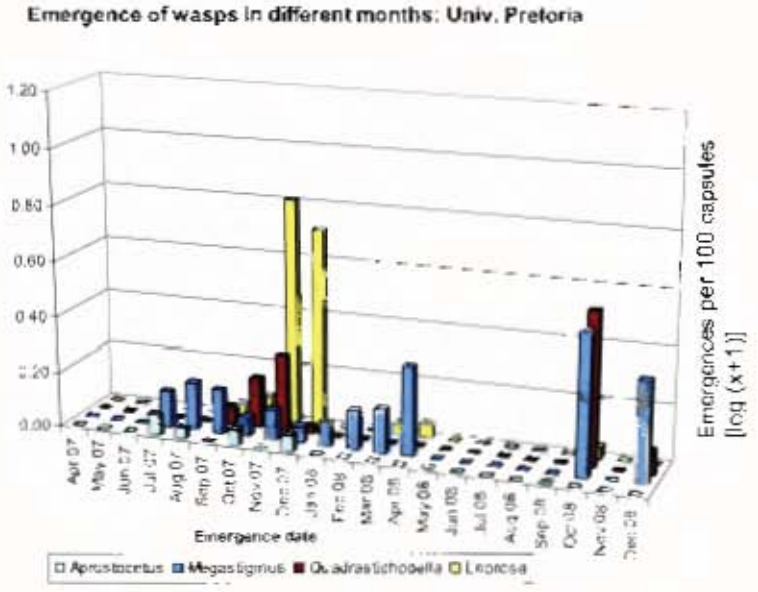

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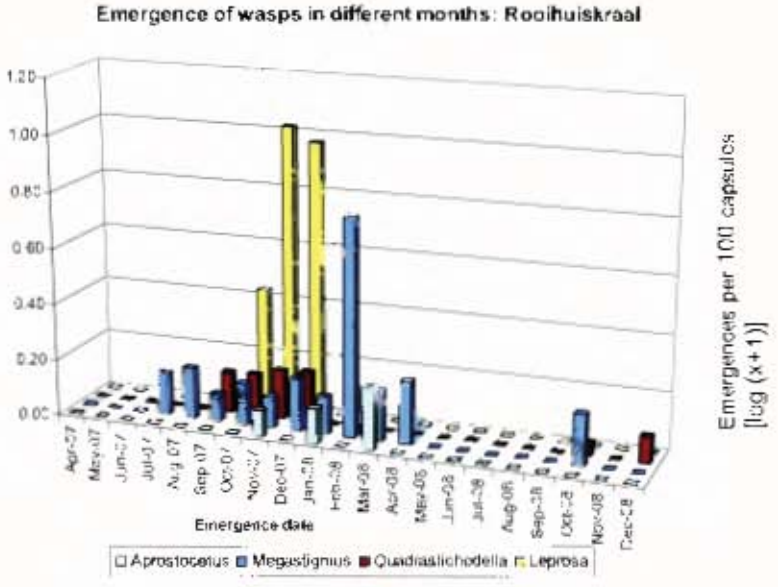


Figure 2.2 Emergence patterns of wasps from seed capsule samples collected from three *Eucalyptus camaldulensis* trees near Pretoria

Regarding emergence numbers, there were significant differences between sample sites in the emergence of *Q. nova* (H = 6,48; df = 2,54; P = 0,039) and *Aprostocetus* sp. (H = 12,32; df = 2,54; P = 0,002) while the emergence numbers of *M. zebrinus* (H =4,15; df = 2,54; P = 0,125) and *L. milga* (H = 2,17; df = 2,54; P = 0,338) did not differ significantly between sample sites. These differences were most obvious between samples collected from the Rietondale tree on one hand, and samples from the other two trees on the other hand. During the first summer (2007/8), *Q. nova* emerged as the most abundant species (total 6,5 per 100 capsules – table 2.3) from the Rietondale samples, followed by *L. milga* (total 3,06 per 100 capsules – table 2.3). The opposite was true for the other two trees, where samples yielded *L. milga* in greater numbers (total 3,9 and 4,46 per 100 capsules for University of Pretoria and Rooihuiskraal samples respectively– table 2.3) and *Q. nova* in far smaller numbers (total 0,63 and 0,41 per 100 capsules for University of Pretoria and Rooihuiskraal samples respectively– table 2.3) than the Rietondale samples. The numbers of *Aprostocetus* sp., although lower in total than those of *Q. nova* and *L. milga*, were significantly higher in the Rietondale samples (total 0,79 per 100 capsules– table 2.3) than in samples from the other two trees (total 0,08 and 0,05 per 100 capsules for University of Pretoria and Rooihuiskraal samples respectively– table 2.3). *Megastigmus zebrinus* emergence numbers were lower in samples from Rietondale (total 0,3 per 100 capsules– table 2.3) than in samples from the other two trees (total 0,74 and 1,1 per 100 capsules for University of Pretoria and Rooihuiskraal samples respectively– table 2.3).

Although no new seed capsules were collected after March 2008, *Q. nova* and *M. zebrinus* continued to emerge from samples collected from all three test trees during the second summer (2008/9), with *L. milga* emerging from all except the Rooihuiskraal samples; no individuals of *Aprostocetus* or Eulophid #4 emerged from any of the samples during the second summer. In samples from both Rietondale and University of Pretoria, *Q. nova* emerged in greater numbers than *L. milga* during the second summer, with the numbers of *Q. nova* from University of Pretoria being considerably higher during the second than during the first summer.

Table 2.3. Total number of individuals of different hymenopteran species emerging from combined samples collected from three *Eucalyptus camaldulensis* trees throughout the year April 2007 – March 2008, expressed as number per 100 capsules.

	Megastigmus zebrinus	*Quadrastichodella nova*	*Leprosa milga*	*Aprostocetus* sp.	Eulophid #4	Total
Rietondale	0.3	6.5	3.06	0.79	0	10.65
Univ. Pretoria	0.74	0.63	3.9	0.08	0.02	5.37
Rooihuiskraal	1.1	0.41	4.46	0.05	0	6.02
Total	2.14	7.54	11.42	0.92	0.02	22.04

2.4.2. Monitoring of tree phenology

2.4.2.1 Identification of different developmental stages of reproductive structures (Fig. 2.3): The reproductive structures of trees of the *E. camaldulensis* complex were divided into seven externally discernible stages, stages A to G (Fig. 2.3).

2.4.2.2 Development of tagged clusters of reproductive structures (Fig. 2.4): The duration of each developmental stage on each of the three test trees is shown in figure 2.4. The very youngest flowerbuds (stage A) gave rise to the young flowerbuds (stage B) within approximately 2 weeks, and these matured into fully developed flowerbuds (stage C) after approximately 3 weeks. The fully developed flowerbuds remained in this stage for between 15 and 22 weeks, until the flowers opened (stage D). The duration periods of the flower (stage D), and of the young seed capsules to which the flowers gave rise (stage E), were the shortest of all stages, each at less than two weeks. Either the flowers or the youngest seed capsules, or even both, were frequently missed between two consecutive fortnightly sampling events. The youngest seed capsules, with a sunken, cup-shaped disc (stage E), developed into fully developed seed capsules (stage F), with a bulging disc, which remained in this stage for 14-15 weeks, until the slits between individual valves became visible. The green or brown, woody capsules (stage G), characterised by valves that were clearly divided, remained on the tree for at least 35-40 weeks, before they dropped off, or the branches to which they were attached, were broken off. A large portion of clusters were lost from the tree due to natural causes or sometimes vandalism before the end of the monitoring period.

A. Very youngest flowerbud. Starts when bracts covering the cluster are shed. From 1-2 mm to approximately 3.5 mm long, excluding stalk. Ovules whitish.

B. Young flowerbud. Outer operculum (o.op) shed, leaving scar (sc). 4-5 mm long, including inner cap. Internally: stamens and locules visible.

C. Mature flowerbud. 6-7 mm long, rounded, green to yellow. Hypanthium (hy), scar & inner operculum. Internally: ovary (ov) with ovules and stamens off-white to yellow, placenta light-brown

D. Flower. Inner operculum (i.op) sheds, stamens unfold. Anthers yellow, some more whitish. Stigma (st) moist & green.

E. Youngest capsule. Green or yellow. Style present or absent, stamens shed, disc cup-shaped. Internally: ovules white, undifferentiated.

F. Soft green capsule. Disc (d) bulging, same length as hypanthium (hy), rim (r) brown. 7 mm long, 5 mm diameter. Ovules light brown.

G. Woody capsules. Valves (v) visible. Internally: chaff & seeds yellow-brown, valves dark brown, placenta white with dark edge, wall of capsule light green, skin green.

Figure 2.3. Developmental stages of the reproductive structures of *Eucalyptus camaldulensis*

The duration of the different developmental stages was calculated conservatively. When the youngest flowerbuds (A) were tagged, they could have been several days, or even a week old, which resulted in an underestimate. In calculating the duration of stage G, only those clusters that were tagged in the beginning of the trial as G were included, since for all the other clusters only a small fraction of the time in stage G was observed. Even for those that were tagged as G during April 2007, it was unknown how long they had been in this stage already, and many of them were still present on the tree by the time the trial was ended during March 2008. The calculated duration of stage G was therefore greatly underestimated. If these factors are taken into consideration, the first seven stages (A to F) develop over a period of almost a year, while the woody seed capsules (stage G) remain present for approximately another year. The valves of the woody seed capsules remain closed, with the seeds intact, until the connection between the vascular bundles and the capsules is severed when the capsule drops from the tree, or is removed prematurely. Under those conditions, the valves open and release the seeds, which are mixed with large amounts of chaff (Brooker *et al.* 2002).

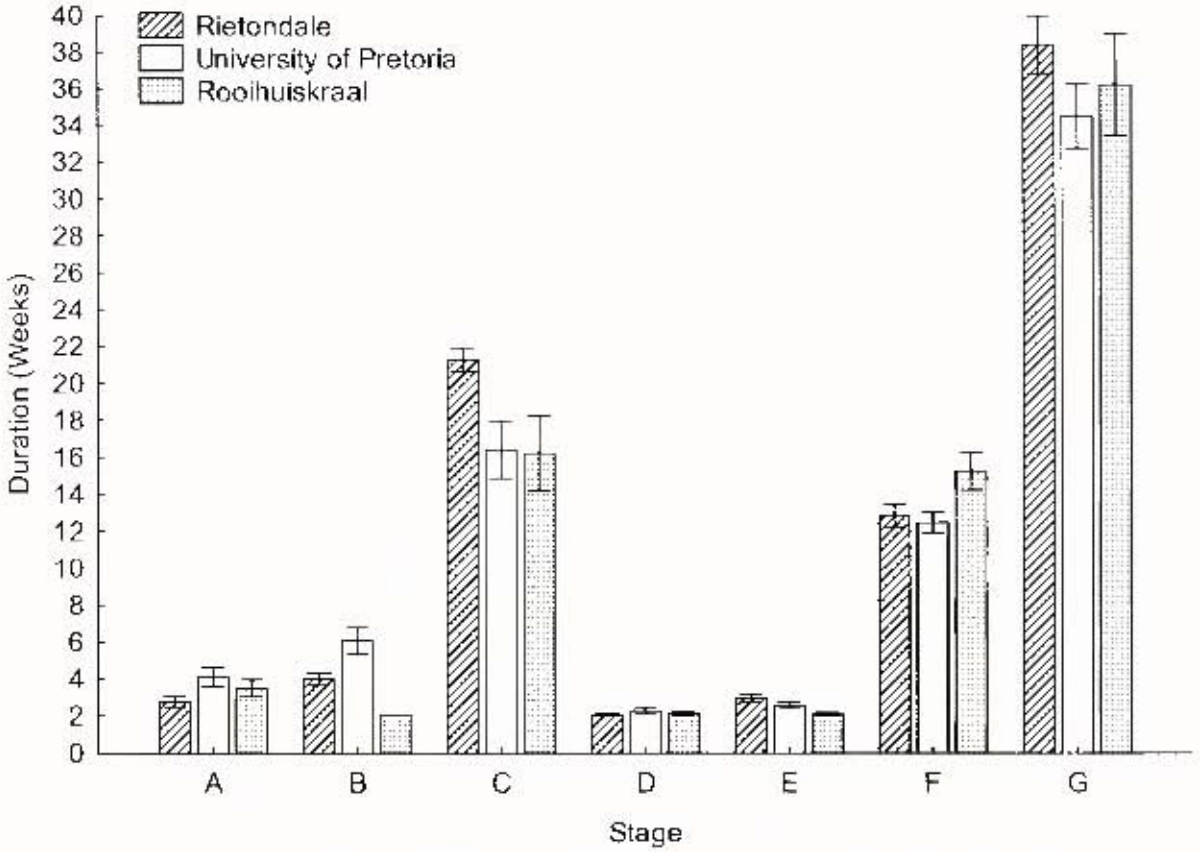
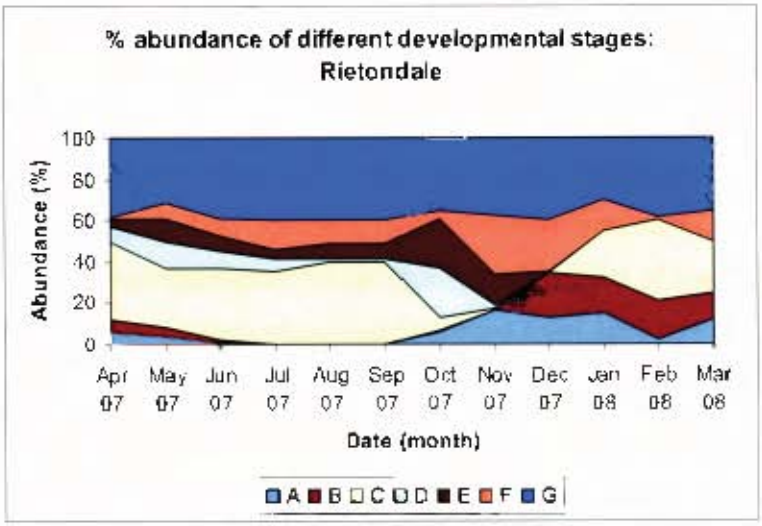
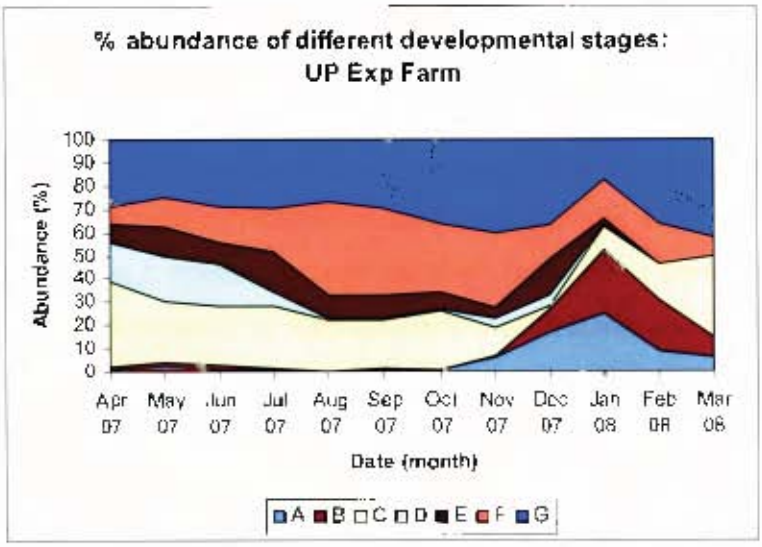


Figure 2. 4. Duration of the different developmental stages of the reproductive structures on three test trees belonging to the *Eucalyptus camaldulensis* complex





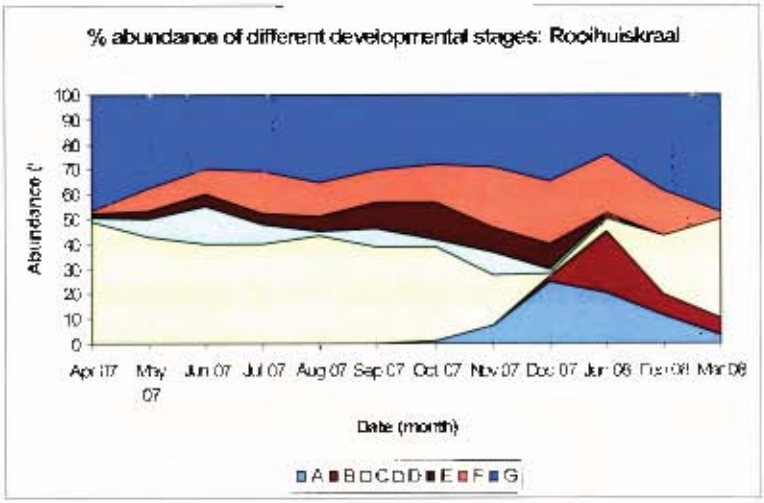


Figure 2.5. The percentage abundance of each developmental stage during sampling events throughout the year April 2007-March 2008 on each of the three test trees (for explanation of developmental stages, see fig. 2.3)

2.4.2.3 Relative abundance of development stages at different times of the year (Fig. 2.5): The proportion of each of the seven developmental stages at any one time is shown in fig. 2.5 for each of the three test trees. The young flowerbuds (stages A and B) appeared during spring (September/October), peaked in mid-summer (December), and were still present in small numbers until winter (June/July) on two of the test trees. On the third tree (Rooihuiskraal), young flowerbuds were absent from April to September. Fully developed flowerbuds (stage C) were present in relatively large numbers throughout the year, except for a short period in November/December, when they were virtually absent from the Rietondale tree, and present in very low numbers on the other two trees. Flowers (stage D) were present in varying numbers from April to November on the Rietondale and Rooihuiskraal trees, but on the University of Pretoria tree there were two distinct flowering periods: a peak from April to August and a smaller flowering period from October to January. The young seed capsules (stage E) had a similar abundance pattern to that of the flowers, but lagged slightly behind. Fully developed seed capsules, both green (stage F) and woody (stage G) were always present in large numbers on all three trees, except for a short period during February (Rietondale) or April (University of Pretoria and Rooihuiskraal) when stage F was in short supply.

2.5 DISCUSSION (Figs. 2.1 & 2.2)

Quadrastichodella nova emerges during a relatively narrow period in early summer, between September and December, with an emergence peak between October and November (fig. 2.2). Results that will be presented in chapter 3 demonstrate that *Q. nova* is the gall inducer. In order for *Q. nova* adults to have been able to emerge from seed capsules collected as early as December 2007 (fig. 2.1 - Rietondale tree), the larvae that developed from the earliest eggs (probably deposited in September 2007) must have had almost completed their larval stage by the time the capsules were removed from the tree, three months later. This can be construed from the supposition that, once the connection between the gall and the transport system of the plant had been broken at the time when the seed capsule was removed from the tree, the gall would not have been able to produce any more nutritive tissue for the larva to feed on. From shortly after December 2007 until August/September 2008, *Q. nova* apparently remained inactive, either in its final larval instar or as a pupa, until the next generation of adults emerged again in August or September 2008.

Results to be presented in chapter 3 illustrate that *Q. nova* oviposits into stages C (the fully developed flowerbuds) and D (flowers). Fully developed flowerbuds (stage C) were present on the trees for most of the year, except for a short period between November and December. Flowers (stage D) were present in relatively small numbers from April to November or December. This implies that, when *Q. nova* emerges, it finds either mature flowerbuds or flowers, or both, in abundance, but both stages became scarce towards the last half of the emergence period. Thus, although the "window of opportunity" for oviposition is relatively large, *Q. nova* is not present in the adult stage to exploit the entire period of availability of stages C and D. It is not clear why the emergence period of *Q. nova* is limited to such a small section at the end of the period during which fully developed flowerbuds and flowers are available on the tree, especially if, as argued in the previous paragraph, some of the larvae must have almost completed their development by December of the previous year, and could therefore have been expected to be ready to emerge earlier than September.

One benefit of an oviposition period that is delayed as long as possible (by having a prolonged quiescent period during the final larval instar or pupal stage) might be that this would limit the period after oviposition in which the wall of the seed capsule remains thin and soft enough to allow penetration by the ovipositor of parasitoids. Judging by the number of parasitoids that have nevertheless managed to become associated with the galls of *Q. nova* in South Africa, this strategy does not seem to have been successful in limiting parasitism or inquilinism. In the event that the parasitoids in this system only managed to overcome this defense relatively recently in evolutionary time, the apparently non-functional synchronization lapse in *Q. nova* might be regarded as the "ghost of evolution past".

Other benefits of a prolonged diapause are that part of the population is protected against mortality caused by e.g. food shortage; that it serves to avoid intraspecific competition in the absence of oviposition sites; or that it could diversify the risks of parasitoid attacks by allowing some individuals to emerge at a time when the parasitoids are not around (Yukawa 2000).

Imperfect synchronization between a gall-inducer and its host plant could be the result of climate change, which affects the insect and the host plant in a different ways, e.g. a cold spring may cause the insects to emerge later, but promote earlier budburst or, in this case, bud development in the host plant (Yukawa 2000).

To gain a better understanding of the interrelationships between the gall-inducer and its host plant, this study would have to be extended over several seasons.

The emergence period of *L. milga* overlaps largely with that of *Q. nova*, but lags slightly behind (fig. 2.2). This emergence pattern would fit that of a parasitoid or inquiline that deposits its eggs into the developing galls induced by *Q. nova*. Using the same argument as for *Q. nova* (in the first paragraph of this discussion) the duration of the larval stage of *L. milga* is probably also around three months, while it spends approximately nine months as a fully developed larva or pupa within the gall.

On the Rietondale tree, where *L. milga* apparently did not suppress the numbers of *Q. nova* very effectively during the summer of 2007, leaving relatively large numbers of *Q. nova* to emerge, both these species emerged again during the summer of 2008 (fig. 2.2). However, in samples from the other two trees, the numbers of *Q. nova* that emerged during the summer of 2007 were far smaller than those of *L. milga*, and in neither of these trees did *L. milga* emerge again during the next summer (fig. 2.2). This was confirmed (fig. 2.1) when none of the samples collected from the UP and Rooihuiskraal trees from December 2007 to March 2008 yielded any *L. milga* adults. The explanation might be that the numbers of *Q. nova* that emerged during the first summer and had been able to induce galls were insufficient to sustain the parasitoid or inquiline, *L. milga*, during the second summer.

The fact that such a large number of *L. milga* adults during the summer of 2007 (in the presence of only few adults of *Q. nova*) were not able to produce a next generation during the summer of 2008, fits the presumption that *L. milga* depends on *Q. nova* galls for its survival, and is unable to induce galls itself.

Samples collected from the Rietondale tree were the only ones that yielded *Aprostocetus* sp. in substantial numbers throughout the year; these samples were also the only ones from which *Q. nova* emerged in larger numbers than *L. milga.* If the assumption that *L. milga* parasitizes *Q. nova* is correct, then it might follow that *Aprostocetus* sp. is a parasitoid of *L. milga*. Where *Aprostocetus* sp. is relatively rare, the *L. milga* population is stronger, and can more effectively suppress the numbers of the gall-inducer, *Q. nova.*

The emergence pattern of *M. zebrinus* presented some apparent discrepancies. Results that will be presented in chapter 3 point towards *M. zebrinus* being a parasitoid of *Q. nova* or *L. milga*, yet *M. zebrinus* starts emerging earlier and continues to emerge until after *Q. nova* and *L. milga*. This means that the females of *M. zebrinus* will have to delay oviposition for several weeks until larvae of *Q. nova* or *L. milga* are present to be parasitized, or else oviposit before the host species.

If the latter is the case, the egg or young larva of *M. zebrinus* would have to undergo a dormant period, or the young larva would have to feed initially on gall tissue and only start parasitizing the host larva once the host larva has grown large enough to support the development of the parasitoid. Evidence that will be presented in chapter 3 shows this to be a distinct possibility.

It is also possible that the *Q. nova* or *L. milga* larvae, developing from the first eggs of the season, are so heavily parasitized by *M. zebrinus* - before the thickening seed capsule wall deters any further parasitism - that virtually none of these early *Q. nova* or *L. milga* larvae emerge as adults. This would result in an apparent delay in the emergence of *Q. nova* or *L. milga*, relative to that of *M. zebrinus*, during the next season. However, this still does not explain where the early *Q. nova* or *L. milga* adults will come from during the following season, to produce eggs that can be parasitized by the early-emerging *M. zebrinus* females. Also, with its long ovipositor, *M. zebrinus* is the species most likely to be able to deal with a thick capsule wall. If this is the case, another possibility is that *M. zebrinus* oviposits in mature galls in one-year-old capsules, into the quiescent, fully-developed larvae or pupae of *Q. nova* or *L. milga*, which are almost ready to emerge.

The inference made from the results in this chapter cannot be used as conclusive evidence of the biologies or interrelionships of any of the chalcidoid species under discussion. A longer study, covering at least two consecutive years, might confirm or refute the suspected relationships based on the relative abundance of different species. In such an event, the phenology information obtained from this study should be valuable in ensuring that further experimental work is timed well enough to coincide with the suitable developmental stages of the insects and their host plant.

The surest way of uncovering the role of each chalcidoid species in the gall is, however, by observing the oviposition behaviour of the adults, dissecting the galls to observe interactions between the immature stages of the chalcidoids, and subsequently identifying the particular immatures. Such an investigation will be the scope of chapter 3.

CHAPTER 3: BIOLOGY OF CHALCIDOIDS ASSOCIATED WITH GALLS IN THE SEED CAPSULES OF *EUCALYPTUS CAMALDULENSIS*

3.1 INTRODUCTION

The literature study (chapter 1) showed that all three of the fully identified chalcidoid species associated with the galls in the seed capsules of the *Eucalyptus camaldulensis* complex (*Megastigmus zebrinus*, *Quadrastichodella nova* and *Leprosa milga*) had been reported as the inducers of galls in the seed capsules of *Eucalyptus* species. When seed capsules of *E. camaldulensis* in South Africa were dissected as part of this study, only one type of gall was ever encountered, but frequently all three of the species mentioned above emerged from the same sample of capsules. It is highly unlikely that three different species, occurring together, would each produce a similar gall in the same organ of the same host plant species. Instead, it can be reasonably assumed that two of the species are not gall inducers but inquilines, parasitoids or predators of the gall inducer or of another hymenopteran associated with the gall.

One or more of the authors who described the three species as gall formers might have mistakenly assumed an inquiline, parasitoid or predator to be the gall inducer itself based on the fact that they found only one insect species in, or emerging from, the galls. Failure to discover the other gall inhabitants could have been due to sampling at the wrong time of the year; to not dissecting the galls and therefore not finding juveniles of the other species; or to not matching the juveniles in the galls correctly with the adults that emerged. Information about oviposition was reported for only one of the three species (*Q. nova*), while *M. zebrinus* was described after the dissection of a relatively small number of seed capsules collected in South Africa, and the description of *L. milga* was based on emergence data only. The current study therefore aimed to unravel the biologies of the complex of species associated with the seed capsule galls of *E. camaldulensis* in South Africa.

Very little is known about the biology of gall-making insects and the associated parasitoids, predators and inquilines, because the interactions between them are hidden behind gall tissue (Goolsby *et al.* 2001). At least, most galls occur externally on the plant, which makes it possible to observe them continuously from the early to the final stage, and to dissect them occasionally to obtain information on the developmental stage of gallers, parasitoids and inquilines and the number of inhabitants. Periodical measurement of galls could indicate

the developmental stage and number of inhabitants, and the shape and diameter of exit holes might distinguish emergence of gallers and parasitoids (Yukawa 2000).

In contrast, the galls developing in the locules of the seed capsules of *E. camaldulensis* show no external signs, partially because of the presence of a large amount of chaff in the seed capsule, which can be compressed to free up space for the developing gall. The absence of any external indication of the presence of a gall made it very difficult to collect galls of a desired developmental stage for investigation and made direct observations of the development of the gall and its inhabitants problematic. Instead, indirect methods had to be devised to gain as much information as possible about the galls and their resident hymenopteran complex. X-ray examination of seed capsules (Gupta *et al.* 2004) might have revealed the presence of galls and their inhabitants without the need for dissection, but would have been impracticable in this study, where the galls develop in seed capsules on branches situated several metres above the ground. Since potted eucalypts of any size that will fit into an insectary do not produce any reproductive structures suitable for gall induction, it was not possible to rear the gall associates or to obtain a series of galls of a known age.

Hymenopteran larvae associated with galls are difficult to identify, therefore many studies fail to match the immature stages with their corresponding adults (Shorthouse *et al.* 1990; Manongi & Hoffmann 1995). The most commonly used method for determining matches is to observe immatures in the gall and then to keep them alive until they emerge as adults, which can then be classified taxonomically. According to Goolsby *et al.* (2001) this is the most straightforward method, used widely in the study of gall-inhabiting Hymenoptera. The drawback is, however, that it is time consuming, and not practical in the case of a large suite of parasitoid species. Furthermore, when a gall is removed from the plant, its turgor, humidity and temperature change (Goolsby *et al.* 2001), and the nutritive tissue is not replenished once the gall has been severed from the vascular tissue of the plant, all of which affect the survival of the immature gall inhabitant. The chances of survival diminish even further in the case of a hidden gall, such as the seed capsule gall in *E. camaldulensis*, which can only be detected by destructive sampling. The tough eucalypt seed capsules cannot be dissected on intact branches, three or more metres above the ground, and microscopic observations cannot be made of the minute eggs and larvae inside the galls, before sealing the gall again and expecting the insects to complete their development. Moreover, if a certain interaction is observed between immatures in such a gall, it is virtually

impossible to find another comparable gall of the same developmental stage and with similar inhabitants, which can be held until the inhabitants emerge as adults. It may be possible to remove the larva of an ectoparasitoid from the gall and keep it alive in a humidity-controlled environment by presenting it with fresh larvae of the host whenever necessary (N. Dorchin, Museum Koenig, Bonn, Germany, pers. comm., June 2008). However, larvae that require gall tissue for their development (the gall inducer, any inquilines, and entomophytophagous insects that first ingest the larva of the gall inducer and then continue to feed on the gall tissue) cannot be fed artificially, because of the specialized nature of gall tissue.

An alternative method suggested by Goolsby *et al.* (2001) is to enclose the gall in a sleeve on an intact plant and subsequently collect the emerging insects. This method may exclude species such as hyperparasitoids which arrive in the later stages of gall development. In addition, the lack of any external indication of galling in the seed capsules of *E. camaldulensis* would further reduce the accuracy of this method. However, sleeves can be used to enclose whole branches to exclude free living species and then to add adults of only one species, or more species sequentially. This would enable one to determine which species induce galls in the absence of all other species, and which species require the presence of the eggs or larvae of another identified species in order to complete their development.

It is possible to distinguish between the adults of a gall inducer and the adult of a parasitoid by immediately dissecting the capsule from which an adult has emerged, and searching through the exuviae in the gall to determine whether there are one or two pairs of mandibles among the exuviae (the exuviae are sclerotized and therefore indigestible by a parasitoid). If there is only one pair of mandibles, this would mean that the adult is the gall inducer; if there are two pairs, the adult would be a parasitoid, and the second pair of mandibles would be those of its host (the larva of the gall-inducer) (O. Doğanlar, Ahi Evran University, Turkey, pers. comm. 2008).

The solution chosen by Goolsby *et al.* (2001) was to dissect & observe the gall contents, after which the immatures were matched with adults by means of molecular techniques. Beside saving time (since the adults did not have to be reared before an identification could be made), the biology of immatures could be observed *in vivo* and matched with adults without speculation and without comparison to known biologies of related species (Goolsby

et al. 2001). Partial sequences of the cytochrome b gene (mitochondrial DNA, mtDNA) and of the D2 region of the 28S ribosomal subunit (rDNA) (Auger-Rozenberg *et al.* 2005) were used during that study.

In this study, where the biology of five chalcidoid inhabitants of galls in the seed capsules of *E. camaldulensis* had to be determined, a large number of developing galls were dissected to obtain as much information as possible on the developing hymenopterans and their interrelationships. The intention was to use DNA sequencing to match the immatures with adults of the known species, as recommended by Goolsby *et al.* (2001). In addition, adults were observed in the laboratory, and oviposition trials were carried out on intact tree branches.

3.2 METHODS

3.2.1 Dissection of flowerbuds, flowers and seed capsules (Table 3.1)

Approximately 4000 individual reproductive structures of trees in the *E. camaldulensis* complex, representing different developmental stages, were dissected under a Leica MZ8 dissection microscope. The flowerbuds, flowers and seed capsules dissected in this manner included a minimum of 10 individual reproductive structures of each of the seven developmental stages that were collected from each of the three test trees (discussed in 2.3) at fortnightly intervals for the duration of a year; samples taken to determine the geographical distribution of the hymenopterans (section 2.1); and additional samples of flowerbuds and seed capsules that were collected from other, locally growing, *E. camaldulensis* trees throughout the year.

The observations included the developmental stage of the reproductive structure; the time of the year; the position in which chalcidoid eggs were found; the development of the galls; how and where juvenile chalcidoids developed; how juveniles of different species interacted, and whether any indications of parasitism, predation or inquilinism could be observed. Developmental stages of galls and insects were photographed through the microscope, using a Canon ACK700 digital camera fitted with a microscope adaptor. Video clips were filmed of significant action taking place in the opened galls. The months during which specific juvenile developmental stages were encountered during dissections, as well as the developmental stage of the reproductive structures of *E. camaldulensis* in which they were found, were recorded (table 3.1).

3.2.2 Matching of immature stages with adults of hymenopterans

Two methods were considered for determining the identity of juveniles that had been observed in the galls: a) by matching the DNA sequences of immature stages with those of identified adults (Goolsby *et al.* 2001), and b) by the *in vitro* rearing of juveniles (N. Dorchin, pers. comm.) until the adults could be identified taxonomically. The suggestion by Dr Doğanlar (pers. comm.), retrieving the exuvial mandibles, was received too late to allow its implementation, but it is a sound method and should be used in future, although it will require meticulous dissections of the tiny galls to ensure that all exuviae and mandibles are recovered.

3.2.2.1 Matching DNA of immature stages with adults (Figs. 3.4 & 3.5): Several specimens of the adults of each of the five species that had emerged from collected samples were stored in a 0.6 ml thin-walled, polyethylene PCR® tube with attached cap containing 96 % ethanol; these were kept in the freezer compartment of a refrigerator for future DNA sequencing. Any juveniles that, during dissection, revealed reliable clues to their biology were preserved in the same way. The aim was to match juveniles with adults.

Once such juveniles can be identified, it should become clear which species are phytophagous (e.g. if they are found with green plant material in their gut, or are observed feeding on plant tissue); which species initiate the gall (if their eggs or early-instar larvae are found before the initiation of a gall, or in a gall at a very early stage of development); and which species are secondary inhabitants of the gall (if their eggs or early-instar larvae are found in fully developed galls, with or without the remains of the gall initiator). It should also be possible to determine their relationship to each other (if an egg of one species is attached to a larva or pupa of another species, or if the larva of one species has been feeding on another).

Initially, Dr W. Botha (ARC-PPRI), with the technical assistance of a private company, Inqaba Biotechnical Industries, agreed to examine this aspect by DNA analysis, using sequences from the D2 region of the 28S rDNA genome (fig. 3.4). Due to difficulties with the extraction and amplification of DNA, this collaboration was terminated.

Early during 2008, Dr Bernard Slippers and Gudrun Dittrich-Schröder, a PhD student at the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria,

undertook to complete the work, using the Cytochrome b region of the mtDNA genome to sequence the adults of all five hymenopteran species and those immatures that served as clues to unravelling the biological role of each of the species (discussed in section 3.3.2). Two unidentified morphospecies of chalcidoids that emerged from *Syzygium cordatum* (section 3.3.5) were included as an outgroup. The results are shown in fig. 3.5.

3.2.2.2* In vitro *rearing of immature hymenopterans: Several attempts were made to remove larvae from opened galls and rear them through to adulthood *in vitro* so that they could be identified. The larvae were kept in airtight 0,6 ml polyethylene vials with lids, together with a tiny piece of barely moist paper towel. To keep the larvae from desiccating, yet prevent condensation and subsequent fungal growth in the vial, the moisture had to be controlled carefully, and any drops that formed in the vial were wiped off daily. Some of the larvae were kept singly, while others were presented with a second, live, larva of a different type (at that time it was only possible to distinguish between two types of larvae: a roughly rotund and a more elongated type). The larvae were kept in the vials and daily observations were made until adults emerged or until the larva died.

3.2.3 Laboratory observation of oviposition behaviour of different adults

Whenever sufficient numbers of adults of the three most abundant hymenopteran species (*M. zebrinus*, *Q. nova* and *L. milga*) were available from the emergence boxes (chapter 2), observations were made under a Leica MZ8 stereo microscope of their behaviour when they were exposed in glass vials to segments of eucalyptus stems with flower buds, flowers and seed capsules. Video recordings were made of notable behaviour patterns.

Attention was paid to the following aspects: feeding by adults; the interval between emergence and the onset of ovipositional behaviour; mating behaviour (only applicable to *M. zebrinus*, in which males and females occurred; the other two species were represented by females only); examination of the host plant by adults; interaction between adults of the same and of different species; feeding by adults; probing with the ovipositor; oviposition on flowerbuds removed from branches that had been enclosed in sleeves; oviposition on flowerbuds obtained from exposed branches (i.e. buds that were accessible to other insects before being presented to the wasps under the microscope); oviposition on flowerbuds on which another insect had been seen to oviposit; the depth to which the ovipositor was inserted into a flowerbud, and whether it was adjusted during the process; the number of

times a specific female oviposited into the same bud; whether a female oviposited into a bud into which another female of the same species had already oviposited.

Two newly-emerged females each of *Q. nova* and *L. milga* were dissected and the developmental state of their ovaries examined.

Once oviposition had been witnessed, the flowerbud was dissected to observe the morphology of the egg and the position in which it had been deposited. Such dissections were made at different intervals after oviposition, but the abscised flowerbuds shrivelled after three days, and were no longer suitable for observations.

3.2.4 Oviposition trials in sleeves on trees

To determine which of the chalcidoid species were able to induce galls in the absence of other insect species, oviposition trials were carried out in gauze sleeves on intact branches on trees in the *E. camaldulensis* complex.

On each of the two test trees at Rietondale and University of Pretoria (section 2.3), the distal parts (approximately 200 mm) of selected branches, bearing very young flowerbud clusters (stage A), were cleared by hand of any visible insect pests. The branch section bearing the young buds was covered by a nylon gauze sleeve, which was tied with a string at both ends to enclose the flowerbuds. The aim was to exclude all insects until the onset of the experiment.

When adults of *M. zebrinus*, *Q. nova* or *L. milga* were available from the emergence boxes, but no less than a month after fastening the sleeves in place (when the flowerbuds were fully developed), 2-10 females of a single hymenopteran species were placed into each sleeve, which was then tied up again. In the case of *M. zebrinus*, an approximately equal number of males was added along with the females. Due to the small number of adults of *Q. nova* that emerged from the combined emergence boxes during the time that the flowerbuds in the sleeves were still in a suitable condition, only four sleeves were stocked with *Q. nova* adults. Each sleeved branch was left for at least three months to allow any developing hymenopterans to complete their development, after which the branch was removed and brought to the laboratory, where the sleeve was removed. All flowerbuds, flowers and seed capsules in the sleeve were dissected and examined for the presence of

galls, developing insects, emergence holes or dead insects. Any dead insects in the sleeve were also recorded.

The fact that the results of the insect emergence and tree phenology trials were not yet known when this trial was carried out, resulted in sub-optimal synchronization between the availability of sleeved flowerbuds of the correct developmental stage and adults of the various chalcidoid species. Also, the condition of the sleeved branches deteriorated rapidly, possibly because of heat build-up, but also through infestation of the tree by the hemipteran eucalypt pest, *Thaumastocoris peregrinus*, and the eucalypt shoot psyllid, *Blastopsylla occidentalis* (section 2.3). This reduced the useable number of replicates. Moreover, the second phase of the planned experiment, during which a second chalcidoid species would have been added, a week after the first species had been placed into the sleeve, could not be carried out.

3.2.5 Study of hymenopteran complex in fruit galls of *Syzygium cordatum* in South Africa (Table 3.3)

In order to obtain more information on *Megastigmus zebrinus,* several samples of fruits of the indigenous waterberry, *Syzygium cordatum* (Myrtaceae) were collected and placed into emergence boxes to allow insects to emerge from the material. This was motivated by reports (National Collection of Insects, Pretoria; Grissell 2006) of *M. zebrinus* being associated with fruit of cultivated specimens of *S. cordatum* at a number of localities in and around Cape Town. Material was collected within the indigenous range of *S. cordatum* (11 sites in Mpumalanga and Limpopo Provinces, although no samples were obtained from KwaZulu-Natal, which also falls within the natural range of *S. cordatum*), as well as from four sites in and near Cape Town (outside the natural range of *S. cordatum*) (table 3.3). Several fruits from each sample were dissected to observe where the juveniles developed. Special attention was paid to any specimens of *Megastigmus* that emerged from the samples. One sample of the indigenous *S. guineense* and one of the Australian *S. paniculatum* were also examined.

3.3 RESULTS

3.3.1 Dissection of flowerbuds and seed capsules (Figs. 3.1, 3.2 & 3.3; Table 3.1)

Flowerbuds, in transverse section, showed an ovary comprising between three and five chambers or locules, each of which contained a few ovules (which would eventually give rise to seeds) in the basal part of the ovary, and a large amount of ovulodes (which would

give rise to infertile chaff particles) (Poynton 1979). Both of these were attached to a placenta.

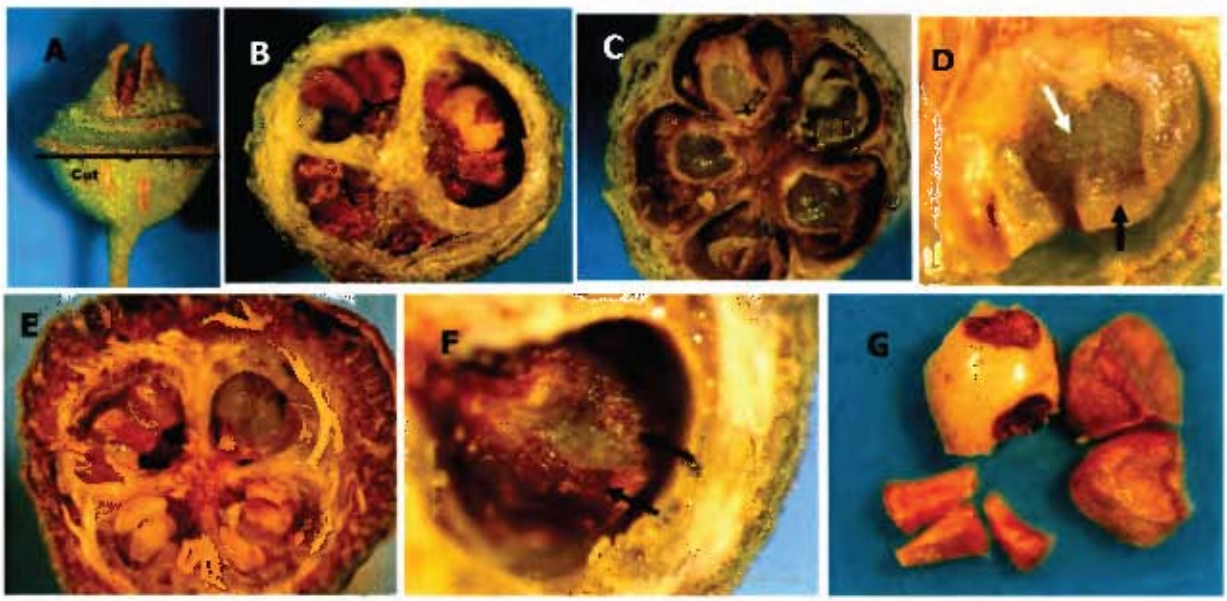


Figure 3.1 Galls in flowerbuds and seed capsules of *Eucalyptus camaldulensis*: A. Seed capsule of *E. camaldulensis*, indicating position of transverse section; B. transverse section of capsule, showing chaff and one gall (top right); C. four galls (opened) in one capsule, in transverse section; D. locule in bud containing young gall (white arrow), with ovule attached distally (black arrow); E. fully developed but still green gall in upper right locule; F. mature gall with two flattened ovules attached (arrows); G. gall (top left), seed (right) and chaff particles (bottom)

Flowerbuds, including those in which females of *Q. nova* had been seen to oviposit, frequently contained insect eggs, attached to or embedded into the placenta by means of a long filament (fig. 3.2 A, B). A day or more after *Q. nova* had oviposited on severed shoots in the laboratory, black marks appeared on the outer wall of the flowerbuds, indicating probing or oviposition sites (fig. 3.2 C). Such marks were, however, not seen on flowerbuds collected from the trees, even though they contained galls. Inside a flowerbud, the oviposition sites were usually marked by brown discoloration in the placenta, and the path of the ovipositor was illustrated by brown marks on some of the ovules and ovulodes between the bud wall and the oviposition site. Similar eggs were never observed attached to, or inserted into, an existing gall, but occasionally one of them formed the centre of a group of enlarged, greenish cells, indicating that they belonged to the gall inducer. In some of the flowerbuds, one or more of the placentas had soft, green protrusions, which were interpreted as representing developing galls, but the egg of the gall inducer was no longer visible. Eucalypt ovules could frequently be seen attached to the outer part of a developing

gall (fig. 3.1 D), indicating that a gall represents an enlarged part of the placenta, and not a modified seed.

When seed capsules were dissected (fig. 3.1 A), between three and five locules were distinguishable, each of which contained a large number of chaff particles and usually one or two seeds. One or more of the locules could contain one or more galls each (fig. 3.1 B). As many as seven galls were observed in one seed capsule, but most frequently, there were from two to four galls per capsule (fig. 3.1 C). The galls were about 1,5 times the size of a seed (approximately 1-2 mm diameter), and roughly spherical (fig. 3.1 G), but could be slightly compressed by the surrounding chaff particles or seeds, which sometimes imprinted their characteristic surface texture onto the gall wall. The walls of young galls were green (fig. 3.1 E) and those of older galls off-white in colour, but had a few brown and completely flattened ovules attached to the outside, which gave the gall wall a mottled brown appearance (fig. 3.1F). The galls were always attached to the placenta, even after all the chaff particles and seeds had been released.

No galls were ever found protruding into the locule from the outer capsule wall, as described by Grissell (2006). However, occasionally a darkened area in the capsule wall formed a protuberance that extended into the locule, but these protuberances were always composed of solid, undifferentiated tissue, without any indication of a gall chamber or any developmental stage of an insect.

In fully-developed capsules (stage F) taken from the University of Pretoria tree, from July to December 2007, very few normal galls were observed during dissection; many of the "galls" contained no insects, but were filled with solid parenchymatous tissue. Most of these capsules had marks in the capsules wall, or outgrowths that extended into the locule. In these capsules, most of the chaff particles were also dark and shrivelled, and they contained no seeds.

When galls in seed capsules were opened, they contained either:

- one of the two types of larvae,
- a larva and an egg,
- two larvae belonging to different types,
- an egg on the inner gall wall, along with a larva or occasionally two larvae
- a pupa, either living or dead

- a living or dead hymenopteran adult, or
- frass and an emergence hole.

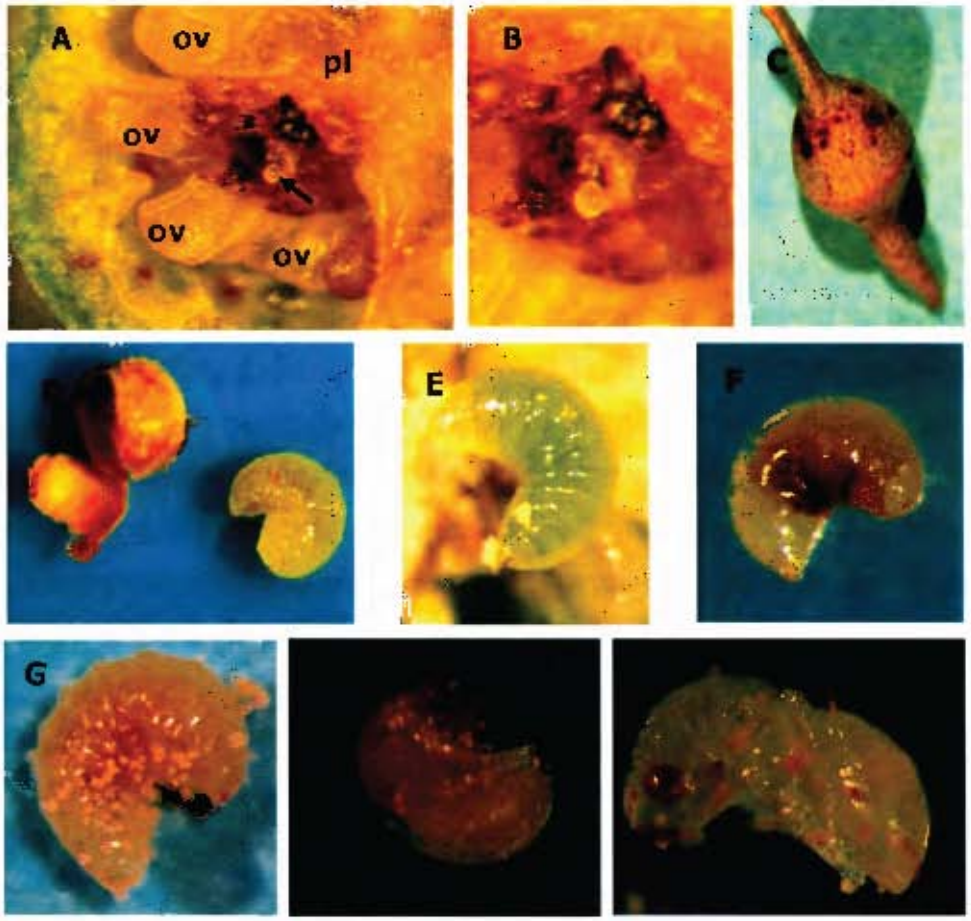


Figure 3.2 Chalcidoid eggs, larvae and pupae: A. Egg (arrow) of *Quadrastichodella nova* in placenta of locule (pl: placenta; ov: ovule); B. Egg of *Q. nova* (close-up) - note filament; C. flowerbud with oviposition and probe marks by *Q. nova*; D. opened gall and fully-developed, "rotund", yellow larva removed from it, possibly *Q. nova*; E. "rotund" larva with green gut content, indicating that it has been feeding on gall tissue, possibly *Q. nova*; F. "elongate" larva with brown gut content, indicating that it has been feeding on a dead larva; G. larva covered with frass, possibly *Megastigmus zebrinus*; H. pupa of female *M. zebrinus*, showing exserted ovipositor; I. pupa of unidentified chalcidoid.

Apart from the eggs of the gall inducer (fig. 3.2 A, B), which were only found in flowerbuds and flowers and were almost spherical and attached to a long filament, four types of eggs were observed in galls within seed capsules:

- large, ovoid eggs without filaments, which were found attached to live or dead larvae inside galls (fig. 3.3 D, E, F);

- large eggs on long filaments, attached to the inner walls of galls (fig 3.3 I). These eggs were found during November or December, in capsules of stage F - a few times together with a live "anteriorly pointed" larva, at least once along with two differing larvae in one gall, and once in a gall without any larva;
- rotund, white eggs (fig. 3.3 G) attached to the inside of the wall of a gall containing green nutritious tissue;
- oval eggs attached to the inner wall of an otherwise empty gall (fig. 3.3 H).

Soft, green galls filled with nutritional tissue, and containing a hymenopterous larva – from young to almost fully-developed individuals - were frequently observed in the youngest seed capsules with cup-shaped discs (stage E) (table 3.1). The firm, but still green capsules with bulging discs (stage F) held galls with most of the nutritional tissue already consumed and containing fully-developed larvae, sometimes together with frass. Although the larvae that were present in galls in the seed capsules were all hymenopteriform and at first looked very similar, it appeared as if there were two types of larvae (possibly representing eulophids and torymids respectively):

- Roughly C-shaped ("hunched" or "rotund") larvae, with a pointed posterior tip but a rounded anterior tip, with mandibles facing each other, and without visible setae on the body (fig. 3.2 D); some larvae fitting this description were white or yellowish, and others contained green tissue in their gut (3.2 E);
- "Elongate" larvae, which were less "hunched" than the previous type, with both ends pointed, mandibles facing downward, and with a few barely visible setae on the body; the gut might or might not contain green matter (fig. 3.2 F); a similar-looking larva (fig. 3.2. G) was occasionally observed covered in frass.

Table 3.1. Juvenile hymenopterans observed during dissection of flowerbuds, flowers and seed capsules of *Eucalyptus camaldulensis.* (See figure 2.3 for an explanation of plant developmental stages)

Hymenopteran developmental stage	Month during which observed												Plant developmental stage in which found				
	Apr 07	May 07	Jun 07	Jul 07	Aug 07	Sep 07	Oct 07	Nov 07	Dec 07	Jan 08	Feb 08	Mar 08	C	D	E	F	G
Eggs						x	x	x	x				x	x	x		
Young larvae				x				x	x	x	x	x			x	x	x
Mature larvae				x	x		x	x	x	x	x	x			x	x	x
Pupae				x			x			x	x					x	x

Frass or faeces were not found in galls containing young larvae, since the connection between the foregut and hindgut only develops during the final larval instar. Occasionally fully-developed larvae were surrounded with frass, and in at least one of the cases it was assumed that they belonged to *M. zebrinus*, because a pupa of that species (identifiable by the exserted ovipositor – fig. 3.2 H) was found in a capsule of the same batch, with similar-looking frass surrounding it.

Exarate pupae of at least three different types were distinguished. Those of *M. zebrinus* females were easy to identify by their pale body and long, exserted ovipositors positioned over their abdomen, reaching forward to their thorax (fig. 3.2 H). The pupae of *M. zebrinus* males and of *L. milga* were easily confused because of their pale bodies and transverse black bands that were always present on the abdomen of *L. milga*, but only in some males of *M. zebrinus*. Black pupae represented Q. *nova*, *Aprostocetus* sp. or Eulophid #4, but it was not possible to distinguish between these species, especially before they turned black (fig. 3.2 I). Pupae, at least those of *M. zebrinus*, were frequently surrounded in the gall with frass, indicating that they had started preparing an exit hole for the adult insect.

The following evidence of interaction between different hymenopteran species was encountered during dissections: In many instances, two larvae, or a larva and an egg (fig. 3.3 A, B) were found together in the same gall, with the smaller one usually belonging to the "rotund" type, and the larger one to the "elongate" type. This was observed during November in e.g. capsules of stage F from the Rietondale tree. Occasionally, the two larvae were of similar size. In a few instances, a living and a dead larva were seen together in a gall (fig. 3.3 C), and occasionally the live larva could be seen sucking fluid from the dead larva. Once, a live "elongate" larva was observed seeming to feed alternatively on a dead, brown larva and the gall wall. In several galls, a live "elongate" larva was seen together with the dry, flattened remains of another larva. When the early instars of the "elongate" larvae were kept in a plastic vial and presented with larvae of the "rotund" type, one specimen of the "elongate" larva was seen to feed on the other larva. None of the "rotund" larvae were ever observed feeding on "elongate" larvae. Occasionally a living larva, sometimes along with a second, different-looking larva, was seen in one gall, with a hymenopteran egg attached by a filament to the inner gall wall (fig. 3.3 I). Dead larvae, with a large egg (without a filament) attached to them, were found in two different galls (fig. 3.3 D, F).

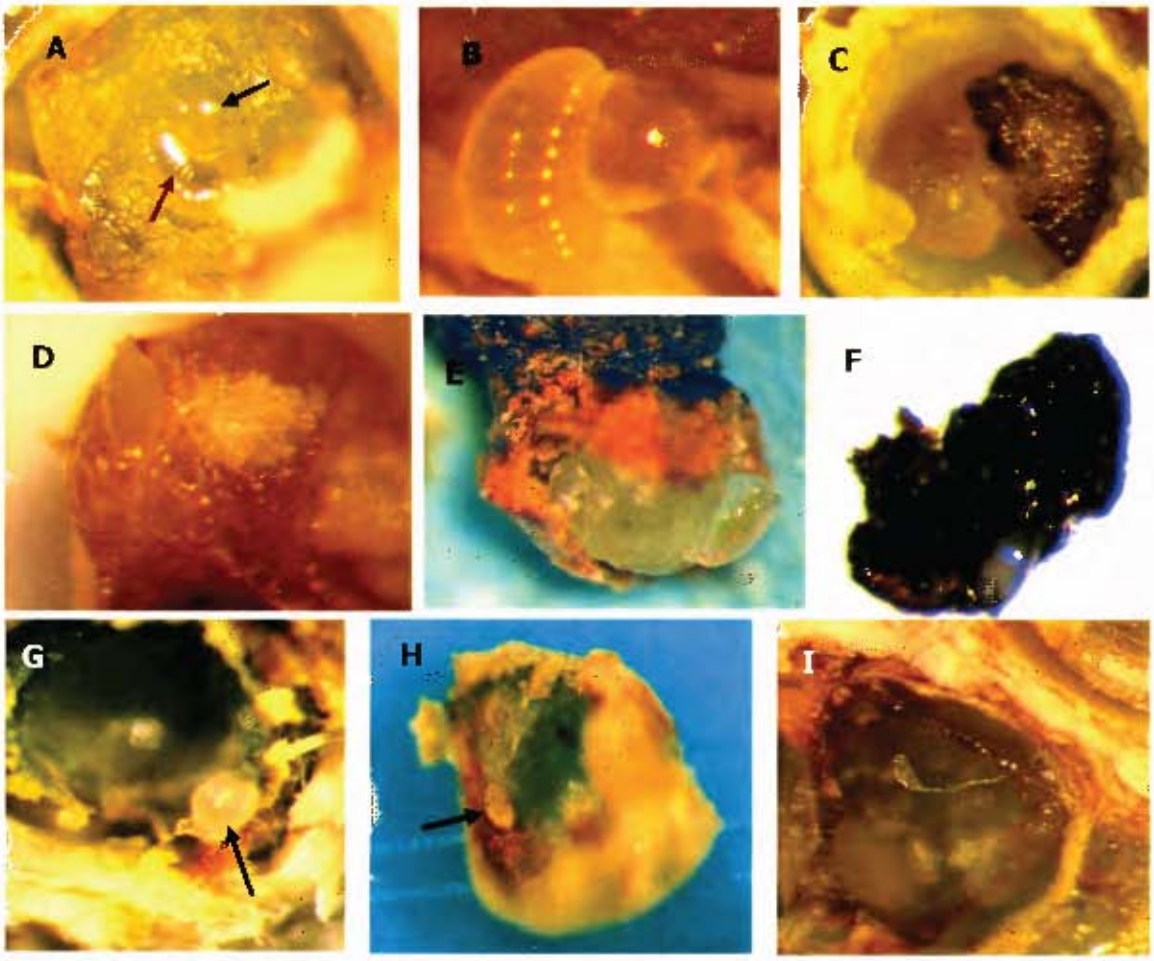


Figure 3.3: Interactions between different chalcidoid species in the galls. A, B, C. "elongate" larva (left) (possibly *L. milga*) feeding on egg or larva (right) (possibly *Quadrastichodella nova*): A. larva and egg intact in gall filled with nutritional tissue; B. removed from gall; C. larva (left) feeding upon dead larva (right); D. large egg or young larva (possibly *Megastigmus zebrinus*) attached to head of dead larva; E. Young larva (possibly *M. zebrinus*) (right) feeding on live larva (left) in opened gall; F. Large egg or young larva (possibly *M. zebrinus*) attached to dead larva; G. Large egg (arrow) attached to inside of a gall containing green nutritive tissue; H. egg (arrow) attached to inside of an empty gall; I. Egg with filament attached to wall of gall that contained two larvae

3.3.2 Matching immature stages with adults of hymenopterans

3.3.2.1 Matching DNA of immature stages with adults (Figs. 3.4, 3.5 & 3.6): Staff of the Inqaba laboratory successfully sequenced the D2 region (28S) for the adults of *M. zebrinus, Q. nova* and *L. milga*. They also obtained sequences for two larvae and a pupa that could be matched with *L. milga,* a pupa that was matched with *Q. nova,* and two pupae that were matched with *M. zebrinus* (fig. 3.4). However, these results did not provide any indication as to the identity of the immature stages of any of the chalcidoid species, because there was no indication of interaction with other species. One larva could not be matched with any of the adults sequenced before, and the closest match on Genbank was

with *Achrysocharoides latreillii.* The laboratory staff was unable to amplify the rest of the samples successfully.

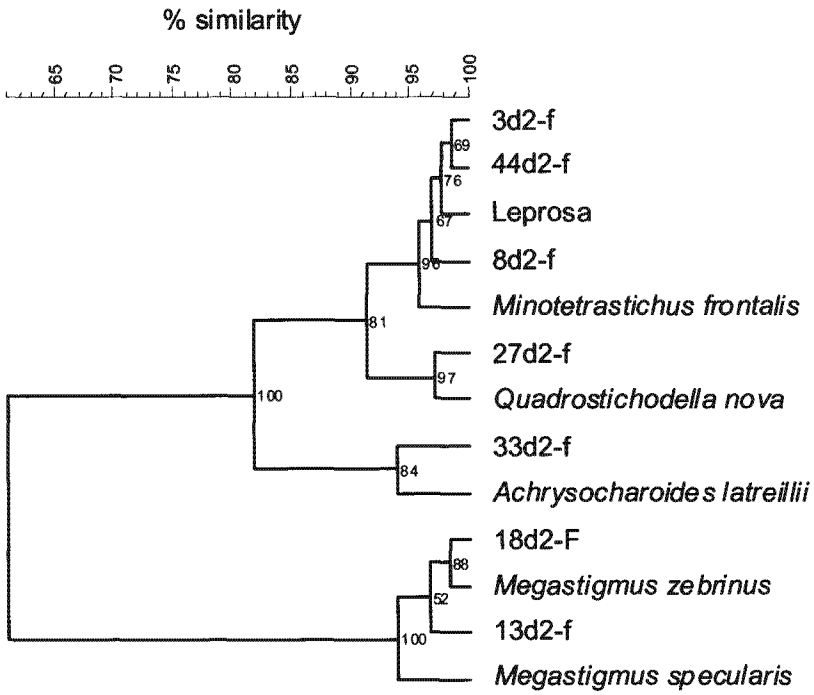


Figure 3.4 Cladogram of chalcidoid adults and juveniles, according to similarities in the D2 region of the 28S rDNA genome, produced by W. Botha

FABI managed to obtain sequences for the adults of *Q. nova, M. zebrinus, L. milga* and *Aprostocetus* sp. using the Cytochrome b region of the mtDNA genome (G. Dittrich-Schröder, unpublished results). Adults of Eulophid #4 were so rare that sufficient material could not be obtained to sequence them. A phylogenetic tree (fig. 3.5) was plotted using these sequences, which clearly confirmed the accepted taxonomic relationships between the four species involved.

Sequences for nine of the ethanol-preserved juveniles have been received from FABI so far, all of which could be matched the sequences of identified adults (G. Dittrich-Schröder, unpublished results). These consisted of one larva of *L. milga* (fig. 3.6 A) (which had been found with another, much smaller larva together in one gall); two larvae of *Q. nova* (fig. 3.6 B, C, D & E), two pupae of *Aprostocetus* sp. and three larvae of *M. zebrinus* (one that had been seen to feed on another larva, and another one with green tissue in its gut). The molecular work is continuing.

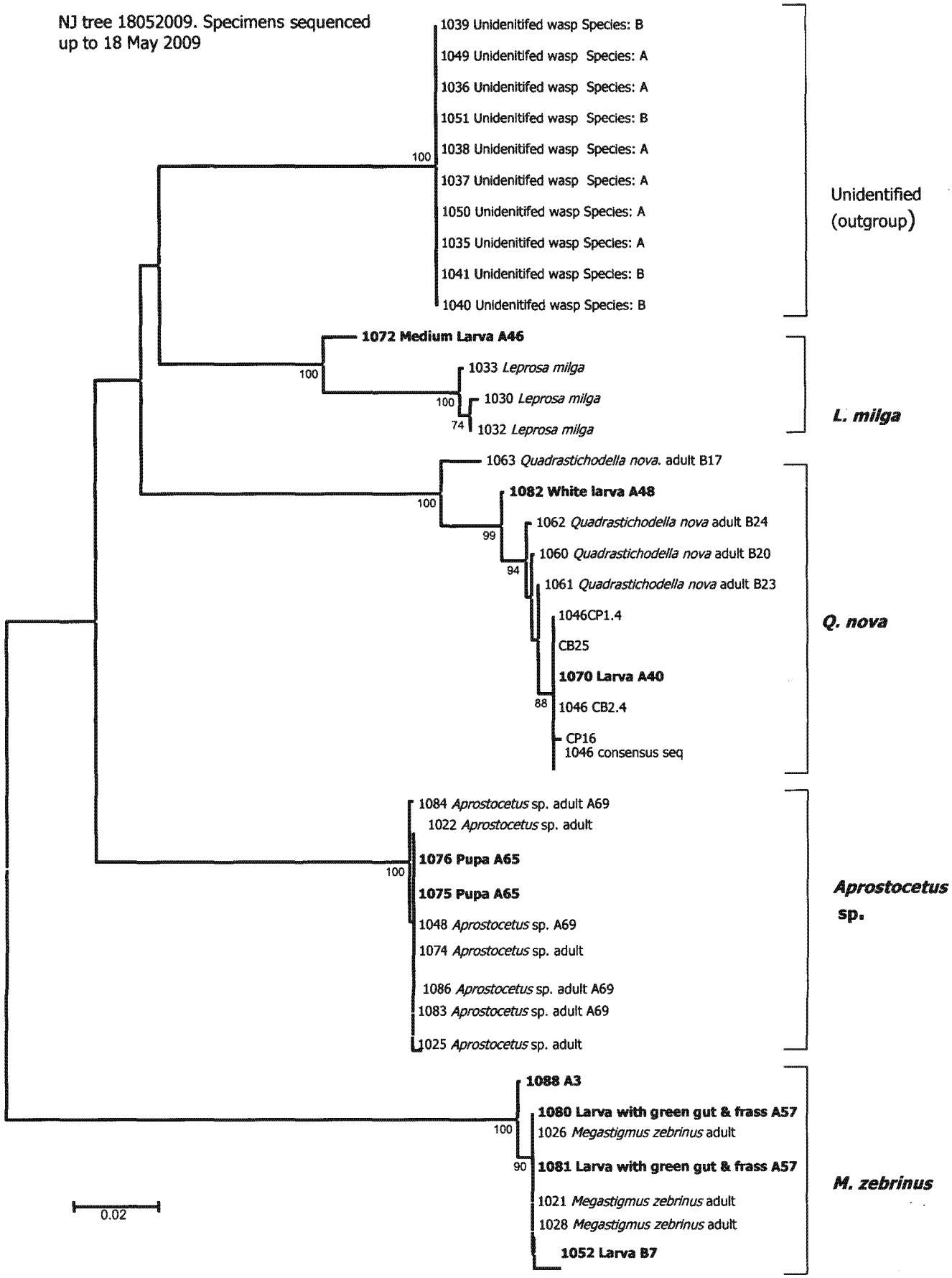


Figure 3.5. Cladogram, showing four chalcidoid species from the seed capsule galls of *E. camaldulensis* in South Africa (produced by G. Dittrich-Schröder, using the Cytochrome b region of the mtDNA genome). Entries in bold indicate juveniles that were matched with adults

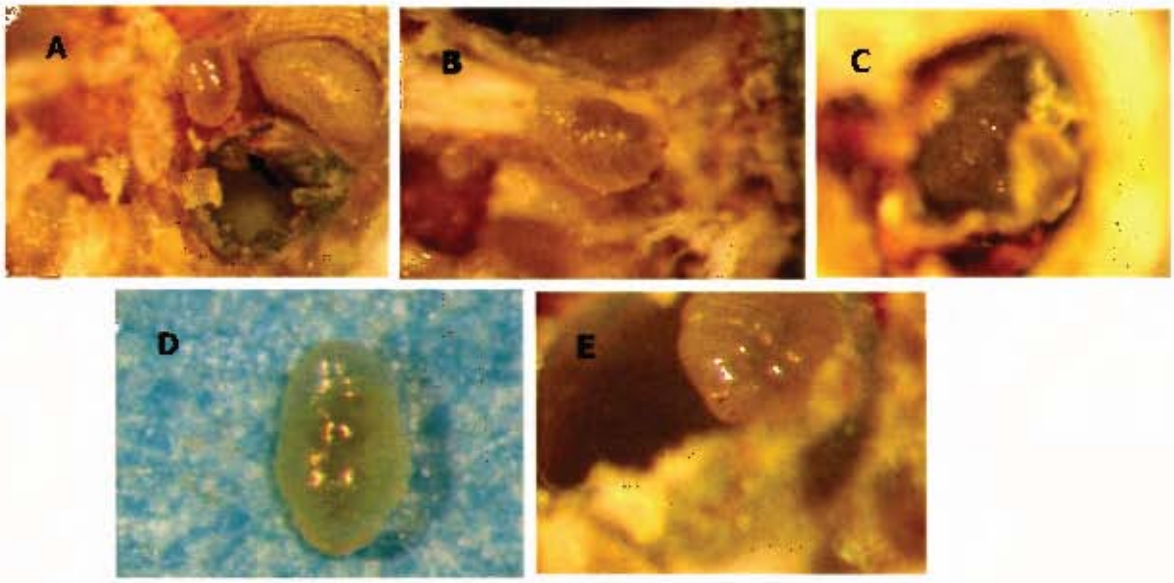


Figure 3.6. Some of the juvenile hymenopterans that were successfully matched with adults by means of DNA sequencing: A. Larva of *Leprosa milga* (left), removed from a gall containing another dead larva (indicated by arrow) and a layer of green gall tissue - note a developing seed, slightly smaller than the gall (right) in the same locule; B. Larva of *Quadrastichodella nova*; C. Larva of *Q. nova* in opened gall. D. The same larva, removed from gall - note green gut contents; E. Mandibles of the same larva

3.3.2.2* In vitro *rearing of immature hymenopterans: It has not been possible to rear through any of the larvae that were kept alive in plastic vials. Most of the larvae were killed by fungal growth that developed despite all attempts to control excess moisture in the vials. The condensation might be attributed to fluctuating day and night temperatures. Those larvae that were not infested with fungus slowly shrivelled and eventually died. This included a few that had been presented with another larva to prey upon. Two of these larvae were seen to attach themselves to the second larva for a short time, and appeared to be feeding, but nevertheless died as larvae.

3.3.3 Laboratory observation of oviposition behaviour (Figs. 3.2 & 3.7)

The females of *Q. nova* were occasionally seen feeding on nectar. As soon as adults of *Q. nova* (from which only females had been observed) were exposed to stem sections of *E. camaldulensis* on the same day that they emerged, they typically started meticulously examining the flowerbuds. This examination included the use of the tip of the abdomen to scrape a substance off the surface of the plant organ, after which the removed substance was passed forward with the legs to the mouth and antennae. Within a few minutes the females started ovipositing in flowerbuds or flowers (fig. 3.7 A). Frequently, one female deposited several eggs in the same bud or flower. While ovipositing, the female sometimes

readjusted the position of her ovipositor repeatedly before the egg was deposited. The entire length of the ovipositor would disappear in the bud (fig. 3.7 B), which indicates that the eggs were positioned towards the centre of the bud where the placenta is situated. Gyrating movements of the abdomen indicated when the eggs were being deposited. It is noteworthy that females of *Q. nova* oviposited into buds that had been sleeved and therefore never exposed to other insects.

When buds into which *Q. nova* had oviposited, were dissected after a day, newly-deposited eggs, each attached to the placenta by a long filament, could usually be detected (fig. 3.2 A, B). At this time, the placentas with eggs had not yet started transforming into a gall.

Dissection of *Q. nova* females showed that all the eggs in the ovaries were fully developed at the time of emergence.

Adults of *L. milga* (which were also represented by females only) were not as active as those of *Q. nova.* Initially they spent much of their time feeding on nectar and pollen (fig. 3.7 C) if they were presented with eucalypt stem segments bearing open flowers. They were often observed piercing the surface of a flowerbud with the tip of their abdomen and turning around to either feed on the exudate or examine it. For several minutes, they sat motionless on a flowerbud, with their antennae held stiffly upright (fig. 3.7 D). In a few cases, but never on the day of emergence, females inserted their ovipositors into flowerbuds or flowers up to about half the ovipositor length (fig. 3.7 E), but not for as long a period or with as much attention to positioning as the females of *Q. nova.* If eggs were indeed deposited, they could not have been positioned in the placenta, but possibly in a gall, closer to the outer edge of the flowerbud. No eggs, apart from those of *Q. nova*, were observed when dissecting the buds, but it was not always possible to recognize the specific bud in which *L. milga* was assumed to have oviposited; consequently some eggs might have been missed. Females of *L. milga* were not exposed in the laboratory to flowerbuds that had previously been excluded from all other insects, and it was unknown whether the buds or flowers, into which they oviposited, already contained eggs or larvae of other insects. When *L. milga* adults were confined to eucalypt shoots in the presence of *Q. nova* adults, the females of the two species did not interact in any way, e.g. by showing signs of aggression or attraction towards one another. When moving over flowerbuds that had recently been visited by the other species, or into which eggs had been deposited by the other species,

neither of the two gave any indication that they recognized the oviposition sites of the other species or had found the necessary cue for oviposition.

Dissected females of *L. milga* had some fully developed, but mostly undeveloped eggs in their ovaries at the time of emergence.

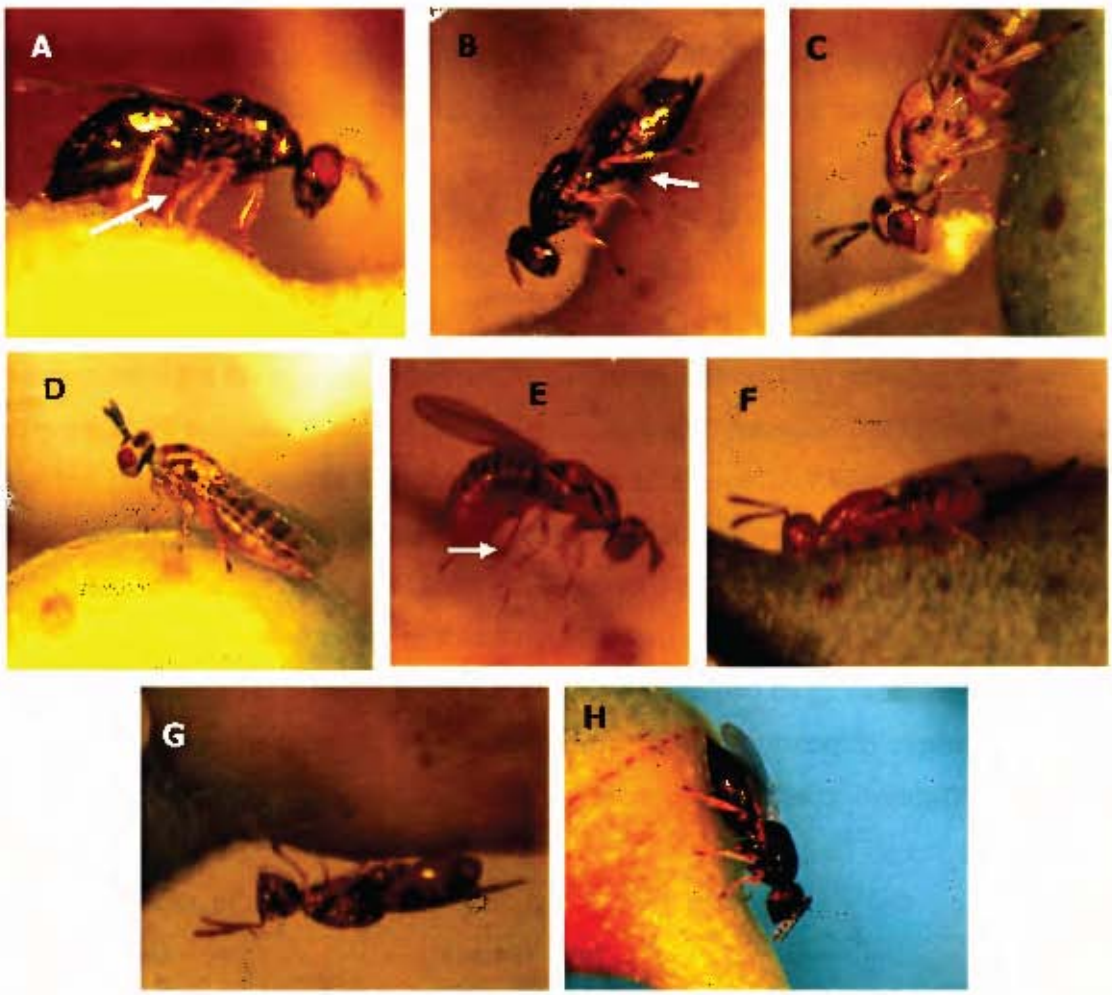


Figure 3.7. Behaviour of chalcidoid species when presented with shoots of *Eucalyptus camaldulensis* containing flowerbuds and flowers: A, B Oviposition in *Quadrastichodella nova*: A, with approximately half of ovipositor inserted into flowerbud, B, with entire ovipositor inserted (white arrow indicates ovipositor); C, D, E *Leprosa milga*: C, female feeding on pollen, D, female sitting attentively on flowerbud, with antennae held erect, and E, ovipositing, with only tip of ovipositor (white arrow) inserted into flowerbud; F, G *Megastigmus zebrinus*: F, female and G, male, both actively examining flowerbuds; H, female of *Aprostocetus* sp. tapping a flowerbud with its antennae

Both the males (fig. 3.7 G) and females (fig. 3.7 F) of *M. zebrinus* were very active, and spent most of their time bustling around on the flowerbuds and flowers, tapping them with their antennae. However, they were never seen to mate, and no female of this species was ever observed ovipositing. The females might have needed older seed capsules with well-developed galls as a cue for oviposition. Females of *M. zebrinus* have not yet been dissected to examine the state of their ovarial development at the time of emergence.

The females of *Aprostocetus* sp. examined the flowerbud systematically, which included palpation of certain regions on the bud (fig. 3.7 H). They have not been observed ovipositing.

3.3.4 Oviposition trials in sleeves on trees (Table 3.2)

The results of these trials are summarised in table 3.2. None of the seed capsules that developed from the flowerbuds in any of the 12 gauze sleeves that were exposed to specimens of *M. zebrinus* only, yielded any galls, and no oviposition marks were visible in any of the capsules. This was also the case for the 17 sleeves that were exposed to *L. milga*. Two of the four sleeves stocked with *Q. nova* did produce galls: one sleeve held one capsule with two galls, one of which was empty and the other one occupied by a dead larva; the other sleeve contained one capsule with two galls, each of which was inhabited by a living, young larva. The latter sleeve also contained two more capsules in which oviposition marks were visible in the placenta. None of the four control sleeves, without hymenopterans, yielded any galls, nor were there any indications of oviposition by wasps.

Table 3.2 Results of sleeve oviposition trials

Wasp species in sleeve	Test tree	Number of replicates*	Results
Quadrastichodella nova	Rietondale	3	Rep. 1&2: No galls or signs of oviposition. Rep. 3: 1 dry capsule with 1 empty gall & 1 gall with dead larva
	Univ. Pretoria	1	Rep. 1: 2 capsules with oviposition marks, 1 capsule with 2 galls, each with young larva
Megastigmus zebrinus	Rietondale	8	No galls or signs of oviposition
	Univ. Pretoria	4	No galls or signs of oviposition
Leprosa milga	Rietondale	8	No galls or signs of oviposition
	Univ. Pretoria	9	No galls or signs of oviposition
No wasps added	Rietondale	2	No galls or signs of oviposition
	Univ. Pretoria	2	No galls or signs of oviposition

* number of females per replicate varied from 2 to 12 and males (only in *M. zebrinus*) from 1 to 10.

3.3.5 Study of hymenopteran complex in fruit galls of *Syzygium cordatum* in South Africa (Table 3.3)

The results of this investigation are reflected in table 3.3. None of the fruits of *Syzygium cordatum*, collected from within its natural range, were galled. No *Megastigmus* spp. emerged from these samples, but an encyrtid wasp was reared from a sample from Nelspruit, and an *Ormyrus* sp. (Hymenoptera: Ormyridae) from a sample from Vaalwater. Apart from these, only lepidopterans and curculionids emerged.

In contrast, most of the fruits collected in and around Cape Town (Cape Town Airport, Pinelands, Rosebank and Fishhoek) had conspicuous, asymmetrical bulges. Dissections showed that one or more seeds of most of the fruits were transformed into multi-chambered galls. Occasionally, single galls occurred on the surface of the fruit. The galls contained mostly one, but occasionally two larvae (apparently from different species), or a larva and the dried remains of another larva. When placed into emergence boxes, these samples yielded a profusion of hymenopterans belonging to at least six species, including small numbers of *Megastigmus zebrinus* (O.C. Neser, National Collection of Insects, Pretoria, pers. comm., Dec 2008) and an exceptionally abundant, black and shiny species in the Anselmellini (O.C. Neser, pers. comm., Dec. 2008). However, *M. zebrinus* was one of the least abundant hymenopteran species to emerge from the galled fruits, and did not emerge from all of the samples.

3.4 DISCUSSION

The essence of the study is that *Q. nova* is the primary gall inducer; *L. milga* is most probably a parasitoid of *Q. nova*, and *M. zebrinus* is a partially herbivorous parasitoid of either *Q. nova* or *L. milga*. *Aprostocetus* sp. might be a parasitoid of *L. milga*, and Eulophid #4 is possibly another parasitoid, but the identity of its host is as yet unknown.

3.4.1 Biology of the gall inhabitants (Table 3.2; Figs. 3.3, 3.5 & 3.6)

Q. nova is a primary gall inducer in this complex. Proof of this is the fact that females of *Q. nova* were observed ovipositing in the laboratory in flowerbuds that had been protected from other insects by a sleeve cage, in addition to the fact that the eggs could be found in those flowerbuds by dissecting them afterwards. This was confirmed by the oviposition trials in sleeve cages (table 3.2): four galls, two of which contained larvae, were found in two of the four sleeves stocked with females of *Q. nova* only, while no galls were found in any of

the large number of sleeves stocked with either *Megastigmus zebrinus* or *Leprosa milga*, or in the controls containing no insects.

Table 3.3. Insects associated with drupes of *Syzygium* spp. in South Africa (* indicates that plant is growing outside its natural range)

***Syzygium* species**	**Collection site**	**Date**	**Results**
S. cordatum	Tzaneen, Witt residence, Limpopo Prov. 23°49'04"S; 30°09' 36"E	Nov. 2004	No galls. Curculionid eggs
S. cordatum	Tzaneen, Debegeni falls, Limpopo Prov. 23°49'01"S; 30°01'56"E	Jan 2005	No galls or emergences
S. cordatum	Tzaneen, Malaria Institute, Limpopo Prov. 23°49'04"S; 30°09' 36"E	Jan 2005	No galls or emergences
S. cordatum	New Agatha State Forest, village garden, Limpopo Prov. 23°56'50"S; 30°08'04"E	Jan 2005	No galls or emergences
S. cordatum	Modjadji's Kloof, Bolobedu village, Limpopo Prov. 23°37'23"S; 30°20'44"E	Jan 2005	No galls or emergences
S. cordatum	Westfalia, Northern Timbers office, Limpopo Prov. 23°44'14"S; 30°06'56"E	Jan 2005	No galls or emergences
S. cordatum	Sabie, Hazyview rd, R536, Mpumalanga Prov. 25°05'38"S; 30°48'38"E	Jan 2005	No galls or emergences
S. cordatum	Sabie, Bergvliet plantation bridge on Hazyview rd, R536, Mpumalanga Prov. 25°04'27"; 30°51'03"E	Jan 2005	No galls or emergences
S. cordatum	Nelspruit Botanic Garden, Mpumalanga Prov. 25°26'36"S; 30°58'13"E	Jan 2005	No galls or emergences
S. cordatum	Near Vaalwater, Limpopo Prov.	Dec. 2006	*Ormyrus* sp. (Ormyridae)
S. cordatum	Vygeboomfontein, Limpopo Prov. 24°18'26"S; 27°42'28"E	Jan. 2007	No galls or emergences
S. cnrdatum	Marakele, Limpopo Prov. 24°27'40"S; 27°35'58"E	Jan. 2007	No galls or emergences
S. cordatum	Nelspruit, Mpumalanga Prov. 25.28S; 30.58E	Jul 2007	1 encyrtid wasp; 1 black wasp w long black antennae
S. cordatum	Sabie/White River, Mpumalanga Prov. 25°11'51"S, 30°56'27"E	Jan 2008	1 mymarid, 1 scale parasitoid (*Scutelista* sp.)
*S. cordatum**	Rosebank, Cape Town, Western Cape Prov. 33.57S 18.28E	Apr 2008	*M. zebrinus*; Anselmellini; morphosp. 1 (yellow & black bands); Morphosp. 2 (yellow w tip of abdomen black); morphosp. 3 (large brown Torymid); morphosp. 4 (black, not shiny)
*S. cordatum**	Cape Town Airport, Western Cape Prov. 33°58'07"S; 18°35'31"E	May 2008	*M. zebrinus*; morphospp. 1&2
*S. cordatum**	Pinelands, Cape Town Western Cape Prov. 33°56'11"S; 18°30'23"E	Aug 2008	*M. zebrinus*; morphospp. 1 & 2
*S. cordatum**	Fish Hoek, Western Cape Prov. 34°07'55"S; 18°25'20"E	Aug 2008	Morphospp. 1 & 2, Anselmellini
*S. paniculatum**	Tzaneen, Limpopo Prov. 23°49'04"S; 30°09' 36"E	Jan 2005	2 small black wasps with long antennae
S. guineense	Nelspruit Botanic Garden, Mpumalanga Prov. 25°26'36"S; 30°58'13"E	Jan 2005	*Aprostocetus* sp.; *Ormyrus* sp. (Ormyridae); *Pseudotorymus* sp. (Torymidae)

The small number of galls produced by *Q. nova* during the sleeve oviposition tests might indicate that conditions in the sleeves were unfavourable for oviposition, possibly due to modifications to the microclimate (e.g. build-up of heat) inside the sleeves. The fact that the results of the insect emergence and tree phenology trials were not yet known when this trial was carried out, resulted in sub-optimal synchronization between the availability of sleeved flowerbuds of the correct developmental stage and adults of the various chalcidoid species. It would be desirable to repeat this trial, using more replicates in order to obtain results that are more reliable. The knowledge gained during the present study should ensure that, during any future replication, the sleeves are better synchronized with the development of the chalcidoids and the host plant.

The proof presented above for *Q. nova* being a primary gall inducer substantiates the assertions by Flock (1957) and Doğanlar & Doğanlar (2008) that *Q. nova* deposits its eggs in the young flowerbuds of eucalypts, and that this species is the inducer of galls that, from the description in the literature, fit the appearance of the galls studied here. In the account by Flock (1957), the host plant for *Q. nova* (as *Flockiella eucalypti*) was given as *E. umbellata*, and Doğanlar & Doğanlar (2008) were first to report galling by *Q. nova* in *E. camaldulensis* in Turkey, although unpublished host records from *E. camaldulensis* had existed in South Africa ten years earlier.

If *Q. nova* induces the galls in the seed capsules of *E. camaldulensis,* the remaining four hymenopteran species cannot be gall inducers. During the approximately 4000 dissections of seed capsules and buds, galls of various sizes, colours or stages of development were observed, but these were all interpreted as representatives of the same gall type seen at different stages of its development. No clear indications have been found that a second type of gall exists that could have been caused by another one of the hymenopteran species. Consequently, the other four species have to be parasitoids, predators or inquilines.

No evidence was found of a hymenopteran feeding on any other species during its adult stage, nor was there any indication of a free-living larva having moved from one gall to another to feed on more than one host larva. Therefore, no reason exists to believe that any of the four species are predaceous.

The larva of an inquiline could be expected to live in a separate chamber in the gall ("endogall"), distinct from the one inhabited by the larva of the gall inducer. No evidence of

this has been observed in the *E. camaldulensis* galls. An inquiline larva might change the morphology of the gall, which would result in two differing types of galls - something which has not been witnessed during this study either. The adult inquiline might have killed the host larva during oviposition, after which the inquiline larva might have developed in the original gall chamber, but without feeding on the gall inducer larva (see the definition of an inquiline in 2.3). The observation, in several instances, of a flattened, dead larva in the same gall chamber as a living larva, might support this hypothesis; however, it is more likely that any flattened, dead larvae in the gall had been fed upon by another larva. There is therefore no strong case in favour of the existence of an inquiline as part of the hymenopteran complex, although the possibility cannot be excluded that one of the less abundant species of the gall community (e.g. Eulophid #4) might be an inquiline.

The observations made during the dissection of buds, flowers and seed capsules, as presented above, of a) an egg and a larva, b) a live and a dead, brown larva, or two live larvae sharing a gall, or c) of one larva feeding upon another, and feeding alternately on the other larva and the gall tissue, are evidence of one or more parasitoid species preying upon the larvae of the gall inducer or of another species. In many instances one live and one dead larva were found in the same gall, but the dead larva had almost always been flat and dry and clinging to the inside wall of the hollow gall, indicating that its body contents had been sucked out, which would not have been the case if an inquiline had been involved. The feeding by one larva upon both an insect and the gall tissue would fit the explanation that the parasitoid egg had been deposited during the early development of the gall, when the larva of the gall inducer was too small to sustain the development of the parasitoid larva. The parasitoid larva would then need to supplement the nutrition obtained from the host larva by continuing to feed on the gall wall (see the definition of entomophytophagy in 2.3). Such an insect could also be described as a "partially herbivorous parasitoid" (Dr Graham Stone, Institute of Evolutionary Biology, Edinburgh, UK, pers. comm. 10/3/2009). The available information therefore indicates that both *M. zebrinus* and *L. milga*, at least, are parasitoids. The other two gall associates, *Aprostocetus* sp. and Tetrastichinae genus indet., are probably parasitoids too, and some of the eggs represented in fig. 3.3 G, H or I (all of which had been attached to the inside of an existing gall) will no doubt belong to them.

Before the first DNA sequencing results were received from FABI, it had already been assumed that the entomophytophagous species was *M. zebrinus.* In the opinion of E.E.

Grissell (formerly Systematic Entomology Laboratory, PSI, ARS, USDA, pers. comm. 2008), "if [*Q. nova*] is the gall former, then I would bet that [*M. zebrinus*] is its parasitoid and can also feed on gall tissue. The ability to feed on both the gall former and gall tissue is apparently common in torymid parasitoids". This has been confirmed by the fact that two larvae (sample A57), which both had green tissue in their guts, as well as a larva (sample A3) that was collected alive, sharing a gall with another, dead, larva, were matched by DNA sequencing with *M. zebrinus* adults (G. Dittrich-Schröder, FABI, unpublished research 2009). It is still not clear, however, whether *M. zebrinus* parasitizes the gallformer, *Q. nova*, or the equally abundant *L. milga*. The emergence pattern of *M. zebrinus* does not show a close correlation with that of either *Q. nova* or *L. milga*.

Another source of evidence against *M. zebrinus* being a gall inducer in eucalypts comes from the observations made on the drupes of *Syzygium cordatum* in and around Cape Town. From these observations it is apparent that *M. zebrinus* cannot be the gall inducer in that system, since *M. zebrinus* was one of the least abundant species to emerge, and did not emerge from all samples of galled fruits. Hence, also in the case of *E. camaldulensis*, *M. zebrinus* is unlikely to be the gall inducer. The fact that the *S. cordatum* galls and their community of associated insects were found only outside the natural distribution range of *S. cordatum* indicates that the insects associated with the galls are not indigenous natural enemies of *S. cordatum*, but must have colonized the species from a different host – possibly from *E. camaldulensis*, as suggested by Grissell (2006).

Leprosa milga emerged from almost all the collected samples, often in greater numbers than the gall inducer, *Q. nova;* its emergence peak coincided with that of *Q. nova*, but lagged slightly behind. This emergence pattern indicates a close relationship between the two species, typically that of a gall inducer and its parasitoid. *Leprosa milga* is therefore more likely to be a parasitoid of *Q. nova* than of *M. zebrinus.* A larva that had been observed together with another much smaller larva in one gall was matched by means of its DNA with the adults of *L. milga* (fig. 3.5; 3.6 A), although this does not indicate whether it had been the host or parasitoid, and the identity of the second larva is unknown.

Regarding *Aprostocetus* sp. and Eulophid #4, these species emerged from either one or only a few samples of *E. camaldulensis* seed capsules collected in Gauteng and the Western Cape Province (chapter 2). *Aprostocetus* sp. was almost absent from two of the test trees, but emerged in larger numbers than *M. zebrinus* from samples collected from the

Rietondale test tree, which was also the only one of the three test trees that yielded larger numbers of *Q. nova* than of *L. milga.* As discussed in chapter 2, it might be deduced that *Aprostocetus* sp. parasitizes *L. milga*; where it is present, the population of *L. milga* is suppressed, and *Q. nova* emerges in greater numbers. The absence of Eulophid #4 from most of the samples, in addition to the small numbers in which it emerged (if at all), makes it clear that it does not play a key role in the species community in the galls. It has not been possible to link any juvenile hymenopteran observed during dissections with either of these species with certainty.

It is now possible to explain why both *M. zebrinus* and *L. milga* were previously mistaken for the primary gall inducers in the seed capsules of *E. camaldulensis* (Grissell 2006; Kim & La Salle 2008). The results of chapter 2 indicate that the emergence of *Q. nova* and *L. milga* was confined to a short period between September and December, whereas *M. zebrinus* emerged throughout the year, which might explain why Grissell (2006), who collected in South Africa between January and March, did not find adults of the former two species when sweeping. The only instances during this study in which *M. zebrinus* was the only species that emerged from the seed capsule galls at any site was when single samples were collected from that site. Conversely, both *Q. nova* and *Leprosa milga* emerged along with *M. zebrinus* from all three sites in Pretoria that were sampled fortnightly over a period of a year. Furthermore, E.E. Grissell (pers. comm. 2008) did not carry out molecular studies on the juvenile hymenopterans he had found in the galls, and although he assumed that they belonged to *M. zebrinus*, they have not been positively matched with the adults of *M. zebrinus*.

The belief that *L. milga* induced galls (Kim & La Salle 2008) was based on at least one report of seed capsule galls in *E. camaldulensis* (in Stellenbosch, Western Cape, South Africa) from which *L. milga* had emerged as the only insect species. Since publication of that paper, *Q. nova* has also been reared from the same *E. camaldulensis* tree, in the presence of *L. milga*, as part of the present study. The only instances in which *L. milga* emerged from galls during this study in the absence of *Q. nova* was when only a single sample was collected from any site, whereas fortnightly sampling throughout the year at three sites in Pretoria showed that both *Q. nova* and *M. zebrinus* emerged along with *L. milga* from all three sites.

Several questions still remain about the biology of *M. zebrinus*: Firstly, as discussed in chapter 2, its emergence pattern, starting so much earlier than *Q. nova* and *L. milga,* is not what would be expected of a parasitoid of either *Q. nova* or *L. milga.* Unless the adults are very long-lived, they would need to deposit their eggs before those of the gall-inducer. Although it is apparently not impossible for the eggs of a parasitoid to remain dormant for long periods while awaiting the eggs of its intended host to be deposited within reach of its own larvae, the chances of a female *Q. nova* selecting the same flowerbud and locule in which an *M. zebrinus* egg has been deposited earlier, seem very slim. Also, no evidence of this was ever encountered during dissections in the form of eggs that differed morphologically from those of *Q. nova* in the absence of a developing gall. Secondly, the very high numbers of *M. zebrinus* adults that were noticed by Grissell (2006) at Travellers Rest do not fit the role of a parasitoid of the gall inducer, and such large numbers of *M. zebrinus* adults have not been encountered during this study either. Not even the gall inducer, *Q. nova*, has ever been recorded to be present in such large numbers during this study. Thirdly, it is unclear what the identity was of the structures that Grissell (2006) saw protruding into the locule from the outer capsule wall, and that he regarded as young, developing galls.

Not nearly all possibilities have been exhausted for clarifying the biology of the chalcidoid gall inhabitants. The molecular analyses of the juveniles preserved during dissections have not been completed, and it is expected that this technique will supply many of the answers that are still missing. Moreover, the matching of juveniles with adults through a study of the larval mandibles, as suggested by Dr Oguzhan Doğanlar (Ahi Evran University, Kirşehir, Turkey, pers. comm. 17/10/2008), has not been attempted. This advice was unfortunately obtained too late to enable its implementation. However, it is a sound method, and will certainly be used in future.

It is therefore clear that many of the unanswered questions might still be solved by taking this study to its logical conclusion.

3.4.2 The origin and development of the gall (Figs. 3.1 & 3.2)

Dissections of flowerbuds into which female *Q. nova* had been observed ovipositing showed that the gall originates in the placenta of one of the locules in the seed capsule of a flowerbud. Eggs of *Q. nova* were observed buried in the placenta (fig. 3.2 A, B). In

flowerbuds that were left one or two days longer before dissection, the area surrounding the egg had become swollen (fig. 3.1 D).

Older galls were conspicuously green in colour, contrasting with the straw-coloured placenta, and contained a colourless, spherical larva living in soft, juicy, green tissue without a hard wall.

The next developmental stage was a gall that was still green (fig. 3.1 D), but was protruding from the placenta, and to the surface of which 1-3 undeveloped ovules or ovulodes were attached. These ovules or ovulodes, whose point of attachment to the placenta was incorporated into the developing gall, did not develop any further but shrivelled and eventually were represented by thin, brown scales on the surface of the gall.

As the gall increased in size, it protruded further from the placenta until its final size was about one and a half times that of a seed, reaching almost the entire distance from the placenta laterally to the locule wall, but with room left above and, or, below the gall. The gall was roughly spherical, but if several galls developed in the same locule, their shapes could be modified by adjacent galls. A gall might compress some of the chaff particles in the locule or arrest their development, but had no noticeable influence on the size of other seeds in the capsule. As the gall developed, its colour changed from green (fig. 3.1 E) to off-white to light brown, often partially or fully covered by the papery, dark-brown remnants of ovules on its surface (fig. 3.1 F).

During its development the contents of the gall changed from watery, gelatinous tissue into dry, green nutritive tissue, and a larval chamber developed in its centre. Eventually all green tissue would be consumed by the larva, and the gall consisted of a tough, thin, light-brown wall surrounding a large larval chamber.

The galls remained attached to the placenta after the seeds and chaff had been released from the capsule. Adult hymenopterans escaped from the galls through round emergence holes, and from there either through another hole chewed through the capsule wall, or through the open valves of the capsule. No distinction could be made between the emergence holes of the various hymenopteran species. Peak emergence time of *Q. nova* in South Africa was found to be from September to December.

The description above agrees broadly with the description of the development of a gall by Flock (1957): "In the early stages this gall is pale green and soft; it enlarges rapidly, however, and before the anthers have become exposed it has occupied most of the cell and replaced the ovules. Especially in the early stages, the cavity in the gall is much larger than the gall insect. When the gall reaches its final size it is thin but hard and is nearly spherical; in color it is light brown with darker markings. The gall resembles the seed and is about one and one-half times as large in diameter." The "darker markings" in his description must have referred to the compressed ovules or ovulodes attached to the gall.

To summarize, the gall represents part of the placenta, which has become transformed, and not a modified seed. This refutes the view of Kim & La Salle (2008) who refer to "seed galls". It is also obvious that the entire flowerbud is not transformed into a gall, as Bouček (1988) incorrectly quotes Flock (1957): the flowerbud is "...transformed into a hard gall of the size and shape of the tree seed..." , as does La Salle (1994): "The bud is transformed into several galls which are similar in size and shape to a seed...", although the original description by Flock (1957) simply states that the "seed-like gall(s)", "...grow in one of the four cells of the bud". Doğanlar & Doğanlar (2008) state that the larvae "make a gall-like structure by tightly binding 3-4 young seeds", which was also shown to be inaccurate. It is difficult to reconcile the observation by Grissell (2006), that the galls (which he believed to have been induced by *M. zebrinus*) "appeared to originate in the outer locule wall where the disc and valves meet", with the observations made during this study, as no galls were ever found to be attached to the inside of the outer locule wall. In a few instances, solid and irregularly shaped outgrowths were found extending into the locule from the outer capsule wall, but these had no similarity to the galls, and their origin is unclear. They might have been caused by a hemipteran or other insect feeding externally on the seed capsule or the gall inhabitants.

Whether or not the presence of these galls had any effect on the number of seeds that developed in a seed capsule of *E. camaldulensis* will be discussed in chapter 4.

CHAPTER 4: THE EFFECT OF SEED CAPSULE GALLING ON THE PRODUCTION OF VIABLE SEEDS IN THE *EUCALYPTUS CAMALDULENSIS* COMPLEX IN SOUTH AFRICA

4.1 INTRODUCTION

The relevance of the *E. camaldulensis* seed capsule gallers to the *Working for Water* (WfW) Programme – the funders of the present study - lies in the potential of the insects to act as biological control agents against the invasive tree species. The product that WfW desires is an agent that reduces the amount of viable seeds released by *E. camaldulensis*, but that does not affect the valuable attributes of the tree. This chapter discusses the extent to which the seed capsule gallers fulfil this function.

No record could be found on any previous investigation into the seed production, seed biology, seedling recruitment or invasiveness of *E. camaldulensis* in South Africa. Hence, there was no benchmark against which the effect of the seed capsule galls, induced by *Q. nova* in South Africa, could be compared directly.

In its capacity as a source of seeds for export to numerous countries in the world, *E. camaldulensis* has been studied thoroughly in its native Australia (Walters & Geary 1989; Donald & Jacobs 1994; Wallace 1994; Wallace & Fagg 1994). From this literature it is known that eucalypt seeds are borne in woody capsules, together with infertile chaff particles, with the seed generally located near the bottom of the capsules and chaff near the top (Wallace & Fagg 1994). The seeds of *E. camaldulensis* are very small and soft and rapidly lose their viability in natural situations, although mature, dried eucalypt seed can be stored at least six years with little loss of viability (Wallace 1994). As the capsules mature, the seeds abscise from the placenta, while their viablility and potential longevity in storage increase (Wallace & Fagg 1994). According to Wallace (1994), canopy storage of seed is unusual in *E. camaldulensis*; ripe capsules quickly shed their seed and then fall to the ground themselves. In contrast, Jensen *et al.* (2008), who investigated plant communities on the Murray River floodplain in South Australia, found that *E. camaldulensis* retained most of its seeds in a canopy seed bank (a condition known as 'serotiny'). They reported that, in healthy *E. camaldulensis* trees, seed release peaked in summer (December-February), but seed volumes varied cyclically. In water-stressed trees, seed release was up to nine-fold less than in trees with access to sufficient water.

According to West (undated); Poynton (1979); Brooker *et al.* (2002); Meeson *et al.* (2002); and Jensen *et al.* (2008), river red gum - true to its common name - is a riparian or floodplain species in its native Australia, and grows only in situations subject to periodic inundation, requiring water from floods or local rainfall for germination. Seedling recruitment is therefore episodic.

As a forestry species in southern Africa, *E. camaldulensis* was reported to have good and even germination; in South Africa the species was usually established by planting out stock produced in a nursery, but it has also been raised, at least in Zambia, by sowing seed on ash beds after stacking and burning brushwood cleared for afforestation (Poynton 1979).

To investigate the effect that seed capsule galling by *Q. nova* was having on seed production in the *E. camaldulensis* complex in South Africa, capsule samples were collected from three trees in Pretoria, and seed production was compared between capsules without galls, and capsules with a range of gall numbers.

4.2 METHODS (Figs. 4.1 & 4.2)

A sample of 50 green, fully developed but undehisced seed capsules of the *Eucalyptyus camaldulensis* complex, belonging to stage F (section 2.4.2), was collected from each of the three test trees identified in chapter 2 (Rietondale, University of Pretoria Experiment Farm, and Rooihuiskraal). Only capsules without emergence holes at the time of collection were used. The collected capsules were placed individually into envelopes to isolate the seeds originating from each capsule, and the envelopes from each source tree were kept separate. When the capsules had dehisced, the contents, consisting of chaff and seeds, were shaken out or removed with a pin. Each capsule was dissected and examined microscopically for galls, which usually remained attached to the placenta of the capsule. The chaff and seeds were also examined microscopically, and all seeds were collected.

The seeds, which were larger than the chaff particles and differed in shape, could easily be distinguished from chaff particles (fig. 4.1). The shape of the seeds could be described as an angular cone. Only seeds that contained an embryo were counted as viable seeds, and to distinguish between viable and empty seeds, each seed was squashed to reveal the solid, white embryo, if present. For each seed capsule, the number of galls and the number of viable seeds were recorded.

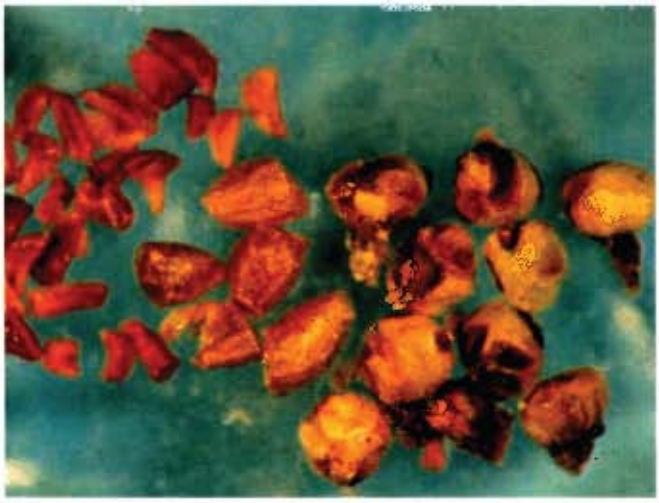


Figure 4.1 Chaff particles (left), seeds (centre) and *Quadrastichodella nova* galls from seed capsules of *Eucalyptus camaldulensis*

The first set of samples from the three test trees was collected during late December 2007 or early January 2008. Another sample was collected from the Rooihuiskraal tree during September 2008, to validate the results of the first collection, which had yielded no galls at all, despite the fact that four of the five chalcidoid species had emerged from galls in the seed capsules of the tree during the investigation described in chapter 2. For comparison, a sample from a tree at Rietondale, growing less than a kilometre from the original Rietondale test tree, was taken at the same time. This tree was chosen because the Rietondale test tree had died early in 2008, probably from infestation by an Australian hemipteran, *Thaumastocoris peregrinus* (chapter 2).

For each of the five samples (three from the first and two from the second sampling occasion), the capsules were divided into categories according to the number of galls they contained; the number of seeds per category (mean ± S.E.) was then calculated. The mean number of viable seeds per capsule was plotted against the number of galls for each of the five samples separately on one set of axes (fig. 4.2).

The five samples were then combined, and the combined sample of capsules was once more divided into categories according to the number of galls they contained. The mean number of seeds per category was calculated and plotted (fig. 4.3). The total numbers of viable seeds per capsule, from all samples combined, were compared between categories containing a) no galls, b) one gall, c) two galls and d) more than two galls per capsule, using a χ^2 test. This comparison could only be carried out for the combined samples, because the numbers of capsules containing three and more galls each were too small to allow statistical comparison within individual samples.

4.3 RESULTS (Figs. 4.2 & 4.3)

When the three sample sites and two collecting times were combined, significant differences were found in the number of seeds per capsule between the category without galls and categories with either one gall, two galls or more than two galls per capsule (χ^2 = 49.915; df = 3; P < 0.0001) (fig. 4.3). The greatest difference was between ungalled capsules and capsules containing one gall, where the mean number of seeds decreased from 0,85 to 0,2 seeds per capsule. More than 34 % of all the sampled capsules fell into either of these two categories. It is therefore clear that the presence of even a single gall could reduce the number of seeds in a capsule to less than a quarter of the number that could be expected in ungalled capsules.

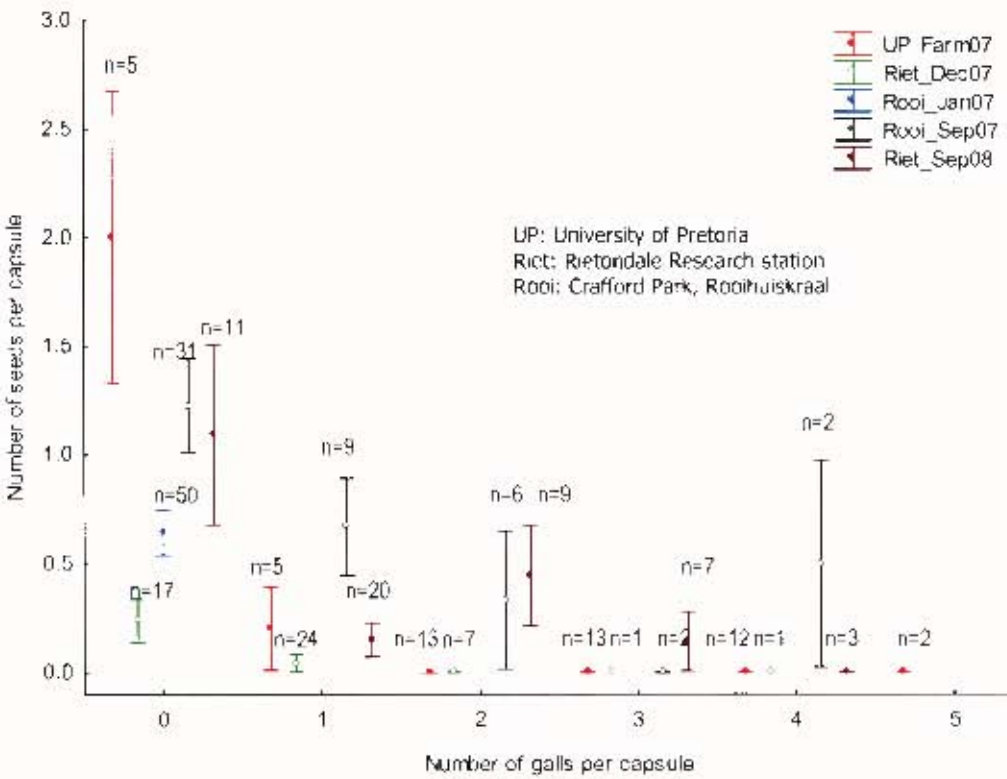


Figure 4.2 Mean number of seeds per capsule in categories containing differing numbers of galls per capsule - Sites separate. Dots and whiskers indicate means and standard error.

When each of the samples was examined individually (fig. 4.2), this pattern became somewhat obscured by the variability in the few seed capsules containing large numbers of galls, although the reduction in seed numbers was still obvious from categories containing no galls to those containing one gall.

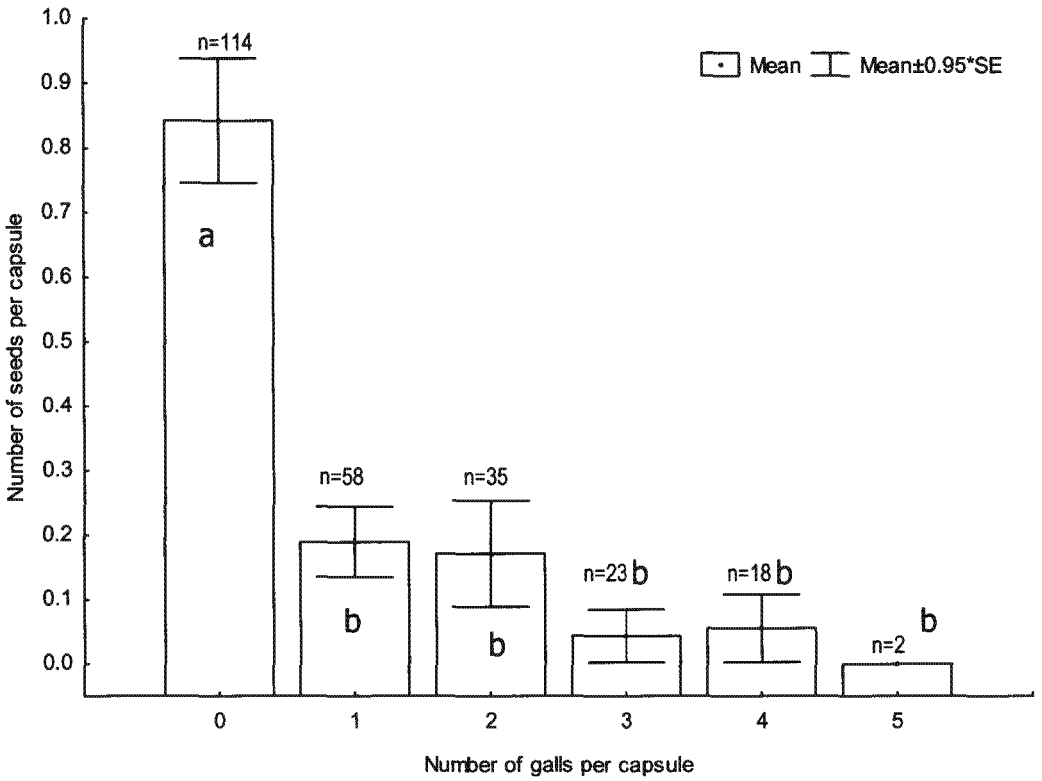


Figure 4.3 Mean number of seeds per capsule in categories containing differing numbers of galls per capsule - Sites grouped together. Bars bearing the same letter do not differ significantly from each other.

The number of viable seeds in the capsules collected during December 2007/January 2008 was very low, even in capsules containing no galls. Ungalled capsules in the sample from Rietondale never contained more than one viable seed (mean: 0,24); those from Rooihuiskraal contained a maximum of three (mean: 0,64) and those from University of Pretoria a maximum of four (mean: 2,0) viable seeds. In contrast, during the second sampling event ungalled capsules from Rietondale could contain up to five seeds (mean: 1,09) and ungalled capsules from Rooihuiskraal up to six seeds (mean: 1,2) (fig. 4.2).

None of the 50 capsules collected from Rooihuiskraal during the first sampling event was galled. In contrast, 66 % of the capsules from the Rietondale sample contained galls, with up to four galls per capsule, compared to 90 % of the capsules from University of Pretoria galled, with a maximum of five galls per capsule, at that time (fig. 4.2). However, during the second sampling event almost 20% of the capsules collected in Rooihuiskraal were galled, with up to four galls per capsule. During the same period, 78 % of the capsules from Rietondale contained galls, also with a maximum of four galls per capsule.

In both the Rietondale and University of Pretoria samples collected during December 2007 an increase in gall numbers was associated with a decrease in the number of viable seeds. The mean number of seeds per capsule was 2,0 in ungalled capsules, and 0,2 in capsules with one gall, in the University of Pretoria sample; the corresponding figures for Rietondale were 0,24 in ungalled capsules and 0,04 in capsules containing one gall. In these two samples, no seeds were present in any capsules containing more than one gall. The second Rietondale sample (not from the same tree as the one sampled during the first collection) showed a reduction in mean seed numbers from 1,09 to 0,15 when gall numbers increased from 0 to 1. However, capsules containing two galls could still have two seeds, and one seed occurred in a capsule containing three galls. Capsules from the second Rooihuiskraal sample containing one gall had a higher mean number of seeds (1,5) than ungalled capsules (1,2), and had up to two seeds. In the same sample, one capsule with two galls still produced two viable seeds, while one seed was even recorded in a capsule containing four galls.

4.4 DISCUSSION

4.4.1 The effect of galling on the numbers of viable seeds (Figs. 2.1, 2.2 & 4.2)
Despite the difference in timing, all the samples (with the exception of the second Rooihuiskraal sample) showed a reduction in the number of viable seeds with increasing numbers of galls in a capsule, at least when the number of galls per capsule increased from 0 to 1. If all samples were combined, a significant decrease in seeds could be detected between ungalled seed capsules and capsules containing any number of galls. Galling in the seed capsules clearly reduces the number of viable seeds that develop in such a galled capsule.

It is unclear why the number of galls in capsules on the Rooihuiskraal tree was so low during the first sampling event during January 2008 (less than one gall per 50 capsules) whereas galls were so much more abundant in the sample taken during September of the next year. The results from chapter 2 (fig. 2.2) showed that adults of the gall-inducer, *Q. nova*, emerged from September to December during 2007, and chapter 3 showed that the females started ovipositing on the day of emergence. By January 2008, some of the larvae should have been three months old. Even though fig. 2.1 (chapter 2) showed that few adults emerged from the December and January samples, while most of the hymenopteran species emerged in relatively large numbers from samples collected during September, the galls should have been visible during dissection, and at least some of them should have

been large enough to have influenced seed production by December or January. The low emergence numbers from capsules collected during those months (fig. 2.1, chapter 2) probably indicate that the larvae still needed to continue feeding in the galls in order to complete their development. The period during which the larva is feeding is in fact the time when the gall reaches its maximum size and when it is acting as a nutrient sink (Brooks & Shorthouse 1998). It could be argued that the Rooihuiskraal tree was still being colonized progressively by *Q. nova*, but the fact that three of the parasitoids (*M. zebrinus*, *L. milga* and *Aprostocetus* sp.) were already present weakens that argument.

If it can be assumed that the reduction in seed numbers in galled capsules is a direct result of the presence of galls, several possible explanations might be proposed for this interaction:

- The developing galls might act as a nutrient sink, competing more strongly for assimilates than the developing seeds. Various authors such as Harris (1980), Freidberg (1984), Dennill (1985); Raman *et al.* (2006) and Veenstra-Quah *et al.* (2007) have shown that heavy galling can act as a strong nutrient sink, reduce seed production significantly and even cause die-back of shoots. It seems unlikely, however, that a lightly galled tree, such as the Rooihuiskraal test tree, could be so seriously affected by the tiny galls in only a small proportion of its seed capsules that seed numbers could have been significantly reduced. The competition for assimilates would have to be very localised, affecting only the seeds developing in the same capsule as the gall.
- Another argument is that the bulky galls might fill up the locules of the seed capsules to such an extent that there is not enough space available for seeds to develop. This is not a satisfactory explanation, because the presence of even one gall in a capsule has been shown (fig. 4.2) to be enough, in certain cases, to prevent the development of any seeds in the capsules; nevertheless, there are usually three to five locules in a capsule, and the locules without galls have sufficient space to accommodate several seeds. Also, the major part of a normal locule is filled with sterile chaff particles, which can be compressed considerably to free up space for the growing seeds and galls, and which could probably be lost without noticeable effect to the plant. Furthermore, as many as three galls (which are usually larger than seeds) have been found in one locule of a seed capsule during dissections, which implies that one gall per locule should still leave sufficient space for at least one or two seeds to develop.

- The position in which a gall is induced - in the placenta of one of the locules of a seed capsule - enables it to tap into the nutrient supply destined for the developing seeds. Most of the vascular tissue of the placenta is apparently transformed into gall tissue, thereby severing the nutrient supply to all the ovules and ovulodes distal to the gall. These ovules and ovulodes would be unable to develop into normal seeds or chaff particles and would, instead, shrivel while remaining attached to the perimeter of the gall. As mentioned earlier (chapter 3), it is indeed a characteristic feature of the galls in *E. camaldulensis* seed capsules that a number of brown "scales", representing flattened ovules or ovulodes, remain attached to the outer wall of the galls. Again, this would only affect seeds and chaff particles in the same locule as the gall.
- Mani (1992) and Rohfritsch (1992) explained how the presence of a gall inducer causes unusual gene expression in the plant cells nearest to the gall-inducing organism, which might alter the physiology and morphogenesis of a minute part of the plant. It is conceivable that, apart from the development of a gall, these changes could have another, as yet undetermined, effect on the plant physiology that could result in a reduction in seed numbers.

The observed seed reduction associated with galling is probably caused by a combination of several factors. The exact mechanism by which it is caused will require a more detailed examination of capsules containing galls of different developmental stages, including histological studies of the placenta, galls and seeds, a study of resource allocation in galled and ungalled seed capsules and other specialised techniques (Lalonde & Shorthouse 1983; Bennett & Van Staden 1986; Hapai & Chang 1986; Van Staden & Bennett 1991; Dorchin *et al.* 2006).

The relatively low number of seeds that were observed even in ungalled capsules might possibly be explained by the fact that none of the test trees grew along a watercourse – the natural habitat of *E. camaldulensis* – and were probably water stressed. Jensen *et al.* (2008) recorded a nine-fold reduction of seeding in water-stressed *E. camaldulensis* trees in South Australia. It is not clear, however, why some of the sampled capsules (both ungalled and galled) contained such relatively large numbers of seeds during September 2008 (before the onset of summer rains) compared to the maximum seed numbers per capsule during the previous December or January (during the rainy season).

4.4.2 The effect of seed reduction on the invasive potential of *E. camaldulensis* in South Africa (Fig. 1)

Considering that this study was motivated by a desire to identify natural enemies of *Eucalyptus camaldulensis* that would reduce the invasive potential of the plant species, any potential biocontrol agent under consideration would have to be evaluated against its ability to diminish seedling recruitment and/or spread in *E. camaldulensis*, according to the concept of invasiveness developed by Richardson *et al.* (2000), in which the rate of spread of seedlings from the mother plant is one of the parameters that determine whether a species is regarded as invasive.

Seed-reducing organisms as biocontrol agents, especially against long-lived perennials, are generally not regarded very favourably in biocontrol circles (Huffaker 1957; Harris 1973; Goeden 1983; Müller 1989; Myers & Risley 2000). Seed losses due to predators or, as in this case, a reduction in seed set due to galling, do not necessarily translate into lower seedling recruitment in a plant population. As discussed by Myers & Bazely (2003), seedling density is determined by seed production, available sites, and seed germination. Hence, recruitment to the population could be limited either by the number of seeds or by the number of microsites suitable for seed germination. Seed reduction due to predation or galling will therefore become a limiting factor in seedling recruitment only if and where sufficient safe sites and suitable conditions for germination exist (Andersen 1989; Louda *et al.* 1990; Maron *et al.* 2002; Meeson *et al.* 2002). For *E. camaldulensis* in South Africa this could be interpreted to mean that seed reduction due to galling will only affect seedling recruitment along watercourses, where conditions are suitable for germination. If no safe and suitable germination sites exist, no seedlings will be added to the population, irrespective of the degree of seed reduction (which would be the case with *E. camaldulensis* trees growing far from water). During a meta-analysis of seed addition experiments, establishment limitation was found to have a stronger influence on seedling recruitment than seed limitation (Clark *et al.* 2007); however, during the same study, seed limitation was greater for large-seeded species (unlike *E. camaldulensis*), species in disturbed microsites, and species with relatively short-lived seed banks (such as *E. camaldulensis*). The storage of seeds, their longevity and their dispersal after release from the canopy are all factors that improve the chances of successful germination (Pettit & Froend 2001). Most models of biological control indicate that 95 to 99.9% of seeds would have to be destroyed before this would reduce plant density (Hoffmann & Moran 1998; Myers & Bazely 2003). This study has shown that galling by *Q. nova* has not yet reached this level of seed reduction in South Africa.

Seedling mortality factors might be as important as seed predation when explaining plant recruitment (Louda 1983). Density dependent seedling survival can compensate to a large extent for limitations on seedling recruitment (Maron & Simms 1997). Certain seedlings, e.g. those of *E. camaldulensis*, are able to resprout from their cotyledon stage, as recorded by Chong *et al.* (2007) during an investigation of resprouting capacity in seedlings from four subtropical, riparian, Myrtaceous tree species. No investigation into the seedling population biology of *E. camaldulensis* has been undertaken in South Africa, and as a result, this aspect cannot be evaluated.

However, it is not only the population density, but also the rate of spread of a plant species that determines how invasive it will become in its introduced range (Richardson *et al.* 2000). South African researchers (Neser & Kluge 1986) demonstrated that the use of seed-attacking organisms had been underestimated as an option in the control of alien plants. They argued that a large seed crop was important in overcoming post-dispersal mortality, and that it also gave the plant an advantage in reaching as many as possible germination sites. Harper (1977) showed that seed loss proportionately reduced the number of seeds reaching any point in the dispersal rate, as well as the distance at which a given density of seeds occurred from the seed source, i.e. the rate of dispersal. Hence, although a reduction in plant *density* was only possible in cases where seed attack reached a specific level, any level of seed reduction would reduce the *rate of spread* of a plant species.

Seed-attackers are likely to have a severe influence in plants lacking seed dormancy, a large, long-lived seed bank and vegetative reproduction; in situations where the insects achieve a high level of attack and maintain it consistently throughout the generation time of the plant; in plant species that have no effective defense mechanisms such as "mast fruiting"; and in situations where the plant populations are homogeneous or where they occur in stable habitats (Kluge 1983, Neser & Kluge 1986). According to these authors, "if prolific seeding is a crucial factor in making a plant invasive, then curbing its seeding even to levels similar to its indigenous analogues, would enable indigenes to compete more successfully" (Neser & Kluge 1986).

It has to be remembered that seed-attackers introduced intentionally as biocontrol agents will be escaping from their predators and parasitoids, as a result of which they are very likely to reach high population numbers in the country of introduction. In the case of the gall inducer in the present study, *Q. nova*, it has been shown (chapter 3) that several parasitic species (e.g. *M. zebrinus, L. milga* and *Aprostocetus* sp.) are associated with it in South Africa. Conversely, the parasitoids might not affect the degree to which *Q. nova* reduces seed set, because these two parasitic species seem to kill the larva of the gall inducer only after the gall has been formed, by which time it might be impossible for the vascular connection to the developing seeds tissue to be restored.

More recently, Richardson & Kluge (2008) showed that, in species with a persistent soil seed bank, preventing the accumulation of seed banks by limiting seed production through biological control is by far the most effective means, and in almost all cases the only practical means, of reducing seed numbers. Andersen (1989) qualified this statement by asserting that, in stable populations of long-lived perennials, seed predation was significant only in species characterised by episodic recruitment events, and that seed predators affected seedling recruitment only if they interfered with the build-up of a seed bank that could take advantage of episodic events conducive to germination. As indicated in the introduction to this chapter, seed germination in *E. camaldulensis* is indeed episodic, but its small, soft seeds are not suited to the accumulation of a soil-stored seed bank, although opinions apparently differ regarding the presence of a canopy-stored seed bank in this species (Wallace 1994; Jensen *et al.* 2008).

In South Africa, *E. camaldulensis* becomes invasive almost exclusively where it grows in or along watercourses, mainly in the Western Cape Province (fig. 1 G). In the few naturally recruited stands of *E. camaldulensis* in inland areas in South Africa, all the saplings seem to be of the same age (fig. 1 F), indicating that they germinated during an episode of unusually wet conditions. This observation fits the pattern of a species whose seedling recruitment is limited by suitable germination sites and episodes, rather than by the amount of seed that is produced. Only those seeds that are released where and when they can be immersed in water for the necessary amount of time will successfully germinate. Where trees grow far from water, a reduction in seed production due to galling will probably have virtually no influence on the invasive potential of the species, as there are no suitable germination sites to take advantage of in any event. Seed reduction may, however, be expected to affect seedling recruitment along perennial watercourses.

Whether this has indeed been happening in South Africa is as yet unknown. Since no surveys have been undertaken of seed production, seed release, seed germination, seedling survival and seed dispersal in *E. camaldulensis* along any of the river systems along which the species is regarded as invasive, it is difficult to determine whether there has been a reduction in any of the mentioned parameters since the arrival of the gall inducer, *Q. nova*, in the country. It is not known how long *Q. nova* has been in the country, and whether there are any significant invasions of *E. camaldulensis* in South Africa that have not yet been colonized by the gall inducer. An attempt should, however, be made to undertake a survey to investigate all of these issues in order to determine whether galling by *Q. nova* is indeed reducing the invasive potential of *E. camaldulensis* in South Africa.

Future surveys for seed-limiting natural enemies of other *Eucalyptus* sp. that are invasive in South Africa, yet are utilized by certain sectors of the population, should bear the results of this study in mind. Cryptic seed-capsule galls with a detrimental effect on seed-production are likely to be present in several *Eucalyptus* species in Australia, and will probably have *Quadrastichodella* species as causative agents. Care should be taken to introduce the gall-inducer, while excluding the associated chalcidoid species, e.g. *Leprosa* spp., *Megastigmus* spp. or *Aprostocetus* spp.. It will be important to complete the current molecular work, by means of which the immature stages can be matched with adults, to enable an early distinction between the larvae of the different species inhabiting the seed capsule galls.

Although the current project has not resulted in the introduction and release of a new biocontrol agent, or even in improved biocontrol, it could still be termed a scientific success in that knowledge has been acquired through scientific investigation to gain an understanding of biological control. In the words of Myers & Bazely (2003): "Even if control is not achieved, an improved ability to predict what will and what will not work in biological control is a measure of scientific success".

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