REPORT FOR ADELAIDE RESEARCH & INNOVATION PTY LTD

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COLLABORATIVE RESEARCH PROGRAM ON THE CONTROL OF THE EUROPEAN WASP IN SOUTH AUSTRALIA

Location of research: Waite Research Precinct, Urrbrae, SA 5064

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South Australian Research and Development Institute (SARDI)
(Department of Primary Industries and Resources SA):

Commencement date: October 1998

Completion date: September 2002

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EXECUTIVE SUMMARY

- The research program on European wasps conducted over 1998-2002 comprised three separate but inter-linked projects that focused on 1) chemical baiting, 2) wasp ecology/population modelling, and 3) prospects for long-term biological control.

- Baiting studies have identified fipronil as a very effective toxicant in a bait for the control of the European wasp.

- Bayer Australia LTD (formerly Aventis CropScience), following collaborative work with SARDI and Landcare Research New Zealand has now focussed on producing a global vespid wasp bait.

- In addition to direct nest destruction, Local Government bodies in South Australia should investigate the feasibility of adding baiting technology as a further control strategy against European wasp. This would be undertaken with a National Registration Authority permit, until a commercial bait product is available.

- Biological studies in the Adelaide region have shown that 1) wasps are more likely to construct nests in the ground than in buildings and aerial locations; 2) the general pattern of nest phenology is similar among seasons but varies in the length of the season between years; 3) light intensity and temperature significantly influence traffic rates in and out of nests; 4) wasps are generalist foragers and respond opportunistically to locally abundant prey and are unlikely to have a detrimental effect on native insect populations, and 5) initial queen weight determines the duration of hibernation and success of hibernation.

- On-going research is now focussing on 1) the population genetics of nests in the Adelaide region using molecular techniques with a view to determining the rate of movement of dispersing queens, and 2) development of a population model to predict the size of wasp populations in the following season. This model will incorporate all available information generated during the three-year study of wasp biology and ecology.

- Classical biological control using the parasitic wasp *Sphecophaga vesparum* was discounted as a viable option given that it has largely proved ineffective in New Zealand and earlier establishment attempts in Australia had failed.

- Extensive field surveys isolated various protozoa, bacteria, fungi and nematodes that were assessed for their pathenogenicity against wasp larvae in laboratory assays. Of these, only nematodes showed any potential and these were examined in more detail. Field trials were conducted on two species, *Steinernema carpocapsae* strain 'Heidi' and *Heterorhabditis zealandica* strain 'Riwaka'. Although the latter species induced approximately 40% larval mortality in the laboratory, neither species proved effective under field conditions.

- Field surveys over three wasp seasons have revealed very little evidence of disease in *Vespula* nests in the Adelaide region. Collaboration with researchers in New Zealand strongly indicates that hygienic behaviour by wasps in their nest and 'antiseptic-like' saliva and venom is possibly the major reason why diseased nests are virtually absent and

pathogens have proved ineffective as control agents in the field. The use of behavioral disrupters in conjunction with pathogens could circumvent this problem but such a strategy would be a long-term endeavour, possibly requiring many years of research.

- In the absence of any likely candidate agents, the wasp gut was examined as a novel source of potential pathogens, as workers forage on a range of bacteria-rich substances that are then passed on to the larva. Denaturing gradient gel electrophoresis (DGGE) proved to be a powerful tool for studying these communities, as it is not constrained by the ability of bacteria to grow on artificial media. Using DGGE, considerable variation in the gut flora of larvae among nests was demonstrated, and a new species was identified which is most closely related to *Rickettsiella grylli*, an insect pathogen. This species has now been targeted by the New Zealand research group as a potential pathogen worthy of further investigation.

OVERVIEW OF PROGRAM

The program was divided into three separate but interactive research projects as follows:

Project 1 Chemical Baiting (undertaken by SARDI)

Project 2 Wasp Ecology/Population Modelling (undertaken by The University of Adelaide, in collaboration with the South Australian Museum and Flinders University)

Project 3 Biological Control Initiative (undertaken by The University of Adelaide, in collaboration with Landcare Ltd, New Zealand)

The outcomes for each project is described in detail in the following pages.

International collaboration

One of the keys to the success of the program was the involvement of a number of high-calibre researchers from overseas. These included Professor Robert Matthews, University of Georgia who is a world expert on the biology of social vespid wasps. He made two visits to Adelaide during the program; one in May 1999 and the other in March 2000. He spent considerable time in the field with Ms Wood, Ms Kasper and Dr Reeson and discussed results with them and potential areas for future research. In addition, Ms Kasper spent two months in Professor Matthews' laboratory during August-September 1999 (the Adelaide winter) to develop skills in field collection of nests and laboratory procedures, prior to being field work in Adelaide in the following summer. Three other key overseas people who collaborated on this program were Drs Jacqueline Beggs and Richard Harris from Landcare Research Ltd New Zealnd, who provided significant advice relevant to all three projects, and Travis Glare from AgResearch Ltd New Zealand who collaborated with Dr Reeson on Project 3.

European Wasp Workshops

An important part of the program was the coordination of an annual national workshop on the biology, ecology and control of European wasps, to facilitate communication among people in Australia. The workshops were held in December of each year of the program, *viz*. at CSIRO Entomology, Canberra in 1999, at the University of Tasmania in 2000, and the University of Adelaide in 2001. These meetings were extremely successful in that they brought together all wasp researchers from Australia and New Zealand, as well as people associated with various state government agencies interested in the management and control of wasps. The workshops covered recent developments in wasp research, and served to strengthen the collaborative links between research teams. In particular the South Australian research team benefited from strong links with colleagues in New Zealand (i.e. Beggs, Glare and Harris), and these are still continuing.

PROJECT 1 - CHEMICAL BAITING

Research officer: Ms Glenys M Wood
Project supervisor: Mr Dennis C Hopkins
Collaborators: Dr Jacqueline Beggs, Landcare Research Ltd, New Zealand
Dr Richard Harris, Landcare Research Ltd, New Zealand
Commencement date: October 1998
Completion date: June 2002*

*Aventis CropScience Pty Ltd has provided funds which have enabled Project 1 - Chemical Baiting to encompass the 2002 season (as discussed with Phil Tyler, 13 February 2001).

INTRODUCTION

Since its introduction into SA in the early 1980's, the main approach to the control of European wasp has been to locate the nest and treat it directly with an appropriate insecticide dust (usually containing either carbaryl or permethrin). Field experience has shown that this approach is very effective but cannot be used in situations where the nest location is not known. Tracking nest locations can be time consuming and very difficult in uneven terrain, so there has been a constant call for additional control strategies to be developed. The most promising alternative to direct nest poisoning was to develop a bait that could be used to reduce the foraging pressure of nuisance populations of wasps and have an impact on the survival of the nest. Nest destruction occurs when sufficient quantities of bait containing an appropriate insecticide are transported back to the nest by wasp workers before the foragers are in turn affected by the insecticide.

In 1996 and 1997, the European Wasp Liaison Committee in SA contracted the South Australian Research and Development Institute (SARDI) to investigate the development of a suitable bait for European wasp (Kitt and Hopkins 1996, Hopkins and Jackman 1997). During these studies, three different insecticides were assessed for use in baits; fenoxycarb, micro encapsulated diazinon and hydramethylnon. No further work was undertaken with the fenoxycarb available in Australia at that time, as the bait/insecticide combination repelled wasps. Both diazinon and hydramethylnon were shown to suppress foraging wasp populations in field trials but as hydramethylnon had no prior registration in Australia for any use, most of the research focussed on diazinon as it was likely to have an easier passage through the registration process. In these studies, it was shown that micro-encapsulated diazinon mixed with protein could be used to suppress populations of foraging worker wasps in a given area for up to 2 to 3 days. However, after this period, significant numbers of worker wasps returned to the same area indicating that this bait treatment had minimal effect on the nearby nests. It was concluded that a more effective chemical was required if baits were to effectively impact on nest survival. During these studies other important outcomes were achieved. Kangaroo mince was identified as being highly attractive to wasps and was considered suitable for use as a bait substrate in any future bait product and a functional bait dispenser prototype was developed to allow wasps access to the bait but excluded all other potential off-target animals and birds.

At the time of the South Australian research, baiting studies in New Zealand identified fipronil, a relatively new insecticide, as promising for baiting of European wasp (Harris and Rose, 1998).

This project, Project 1 in the Office of Local Government's Wasp Initiative, aimed to build on the previous studies to develop a baiting technique that would impact on nest activity as well as reduce foraging wasp pressure. Fipronil and other insecticides were to be fully tested and efficacy packages produced for the most promising chemicals to encourage the respective chemical companies to pursue

full registration for a toxic wasp bait with the National Registration Authority (NRA). The three-year baiting study began in late 1998.

1999 SEASON

Studies in this season concentrated on comparing different concentrations of fipronil with micro-encapsulated diazinon. Three baiting methodologies, measurement of knockdown time, direct nest baiting and broad-area baiting, were used to make an assessment of the efficacy of fipronil for baiting European wasp under South Australian conditions. A slow knockdown time for an insecticide is essential for an effective bait product as this allows the foraging wasps adequate time to transfer enough poisoned bait back to the nest before being knocked down by the insecticide.

Materials and methods

Urban and forest experimental sites with high numbers of foraging wasps were identified using an extensive network of contacts with members of local government and ForestrySA. Two concentrations of fipronil (0.1% and 0.01% by weight) were compared to micro encapsulated diazinon (0.5% by weight). The toxicants were presented to foraging wasps in preservative free kangaroo mince.

Knockdown time of pesticide for wasps

As wasps will faithfully return to a known food source, it was possible to record the visits to, and knockdown times for different strength baits. Foraging wasps were attracted to non-toxic bait (kangaroo mince without insecticide) and individual wasps were marked with model paint with different patterns so that they could be recognised when they returned to the bait. Once wasps were marked, the non-toxic bait was then replaced by the toxic bait and the number of return visits and the time of those visits were recorded. When a marked wasp failed to return to a bait, it was assumed that it had been killed by insecticide in the bait and the time of the last visit was used to estimate the knockdown time. At least 6 individual wasps were monitored for each bait formulation tested (0.1% and 0.01% fipronil and 0.5% micro-encapsulated diazinon).

Direct nest baiting

To reduce the influence of environmental variables and directly assess the action of different insecticides and formulations, 20 g of toxic meat bait was placed directly into wasp nests. The number of wasps leaving the nest per minute was measured before and at 24 and 48 hours after baiting. Nests were baited with three different bait formulations (0.1% used on 4 nests, 0.01% fipronil used on 5 nests and 0.5% micro encapsulated diazinon used on a single nest).

Toxic baiting in broad areas of high foraging wasp pressure

The fipronil baits (0.1%) were tested at two sites with high numbers of foraging wasps. At each site, wasps were attracted to non-toxic baits in bait dispensers (6 dispensers per site) and the numbers of wasps per bait dispenser were recorded using instantaneous counts. After one day, the non-toxic baits were replaced by toxic baits and the counts of wasp numbers were recorded for the following 72hrs. At most sites, non-toxic baits were placed out over the following weeks after the toxic-baiting to assess how many wasps had returned to the area.

Results

Knockdown time of pesticide for wasps

The mean knockdown times of individual wasps for fipronil 0.1%, fipronil 0.01% and diazinon 0.5% were 45.3, 61.6 and 28.1 minutes respectively. These differences in knockdown times were reflected in the number of visits to and from the baits completed by the foraging wasps; fipronil 0.1% had a mean of 5.6, fipronil 0.01% a mean of 8.3 visits and diazinon 0.5% a mean of 4.0 visits.

Direct nest baiting

Fipronil toxic-bait at 0.01% and 0.1% were both more effective than micro-encapsulated diazinon and reduced the wasp nest traffic rate to a mean of <5% over a period of 48hr compared to a reduction to about 30% for diazinon. (Fig. 1).

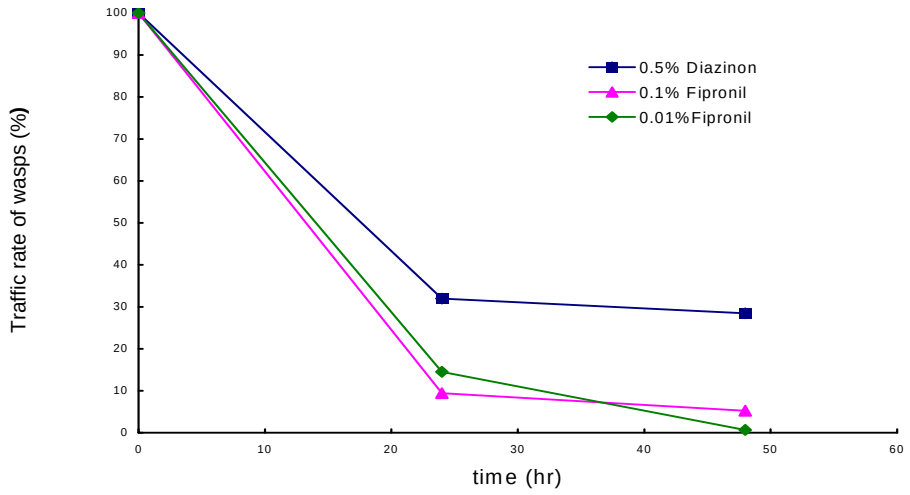

Figure 1. Comparison of fipronil and micro-encapsulated diazinon as toxic baits for European wasp nests

Toxic-baiting in areas of high foraging wasp pressure.

In all sites, the fipronil toxic baits (0.1% at 2 sites, 0.01% at 3 sites) presented in dispensers had reduced foraging wasp numbers by more than 90% after 72hr. At all sites the wasp population remained very low and was considered to be below nuisance levels for the rest of the season.

Outcomes from 1999 research

The results from the knockdown experiments and the direct nest baiting confirmed that fipronil was superior to micro-encapsulated diazinon for baiting wasps. The results from baiting areas of high foraging wasp pressure with fipronil were very promising and indicated that enough bait with fipronil was transported back to the nest, eliminating all wasp activity. On the strength of these results, the research effort for the next season (2000) was concentrated entirely on fipronil baits. It was recognised that fresh or frozen kangaroo meat would be inappropriate as the bait substrate in a commercial product and that there was a need to develop a non-perishable option for any future product.

2000 SEASON

Further trials to establish the best concentration of fipronil in baits were undertaken using fresh kangaroo meat and three concentrations;0.1%, 0.01% and 0.001%. Freeze dried kangaroo meat was also trialed in broad area baiting trials to assess the potential of the freeze dried technique to produce a non perishable bait matrix for the commercial market. Some nest transfer studies were conducted in 2000 to see if small wasps nest could be successfully transferred to artificial nest boxes with a view to using these in bait assessment studies.

Materials and methods

Broad area baiting with fipronil

Three concentrations of fipronil, 0.1%, 0.01% and 0.001%, were tested in fresh kangaroo mince during this season. Three trials were done with 0.1% and 0.01% and a single trial was conducted with 0.001% using the same experimental procedures as used in the 1999 broad area experiments.

Broad area baiting with fipronil in freeze-dried kangaroo meat

20g lots of fresh kangaroo mince were freeze dried and stored in plastic bags at room temperature at the beginning of the wasp season. At the time of the baiting experiments, the freeze dried kangaroo mince was reconstituted with an appropriate volume of water containing sufficient fipronil to produce a 0.1% fipronil bait by weight. These reconstituted baits were then used in broad area baiting trials as above.

Nest transfer studies

Small nests were collected from various sites around Adelaide very early in the nest season and placed in artificial nest boxes (30 x 30 x 40 cm with a hole (4 cm in diameter) in the side). These nest boxes were kept in a shaded, isolated area at the Waite Institute and monitored for continual foraging worker activity over the following weeks.

Results

Broad area toxic baiting with 0.1%, 0.01% and 0.001% fipronil in fresh bait

The 0.1% and 0.01% fipronil baits reduced the wasp foraging numbers to below nuisance level within 24hrs of the bait placement at all three sites and wasp numbers remained low during the following monitoring period of 10 days (Fig. 2 & 3). Fipronil bait at the lowest concentration (0.001%) trialed at one site was unable to suppress wasp numbers for more than 24 hours (Fig. 4) and the experimental site was then successfully rebaited with a 0.1% fipronil bait.

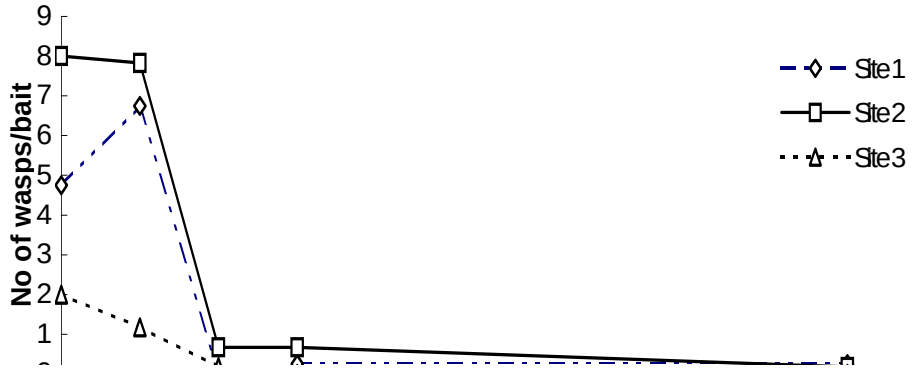

Figure 2. Broad area baiting of European wasps at three different sites with 0.1% fipronil

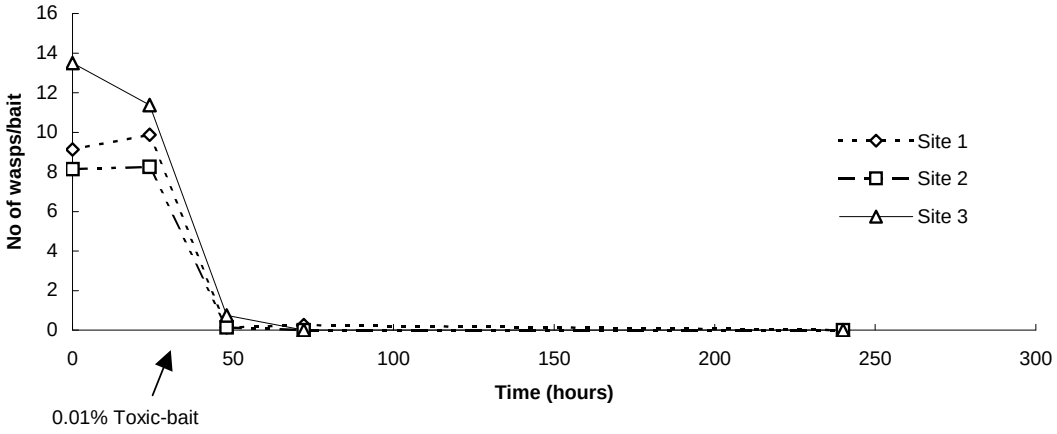

Figure 3. Broad area baiting of European wasps at three different sites with 0.01% fipronil.

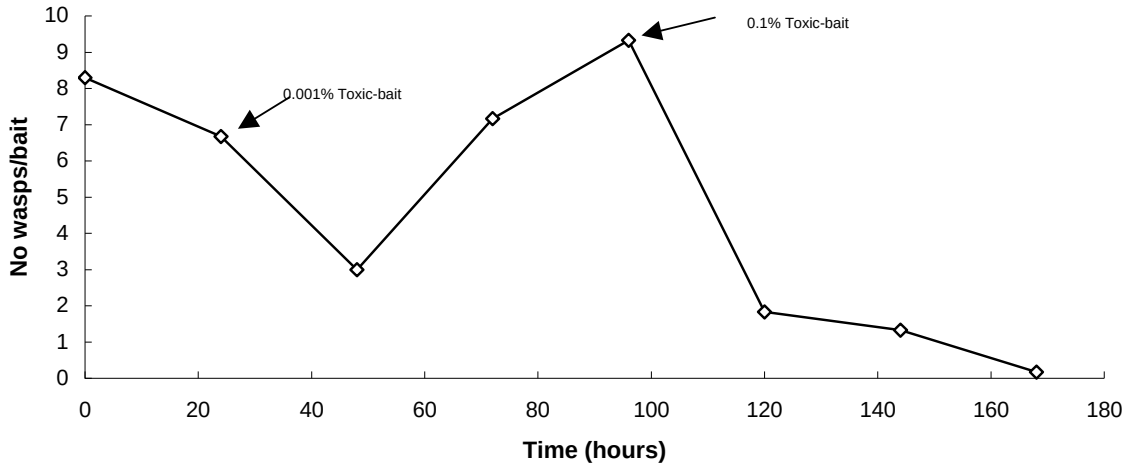

Figure 4. Broad area toxic baititng of European wasps at one site with 0.001% fipronil at Teringie, with follow up baiting with fipronil at 0.1%.

Broad area baiting with 0.1% fipronil in a non-perishable bait matrix 2001

Preliminary trials using the non perishable bait matrix demonstrated the freeze-dried bait reduced foraging wasps as effectively as the fresh meat bait with the same fipronil concentration (0.1%). The wasp activity remained suppressed as the wasps in the source nest were also destroyed. The non-perishable bait matrix used had been stored for approximately 6 months and this process did not appear to impair the attractiveness of it to the wasps.

Nest Transfer Studies

Three early stage nests were successfully excavated and relocated into wooden nest boxes. All three colonies survived and wasp numbers increased over several months. Two nests were used in a baiting experiment and the third declined in numbers probably as a result of extended hot weather. While this nest transfer technique proved to be feasible, it was time consuming and there were difficulties in finding suitable sites to relocate the nests. It was decided that future baiting trials would focus on areas where "natural" nests occurred.

Outcomes from 2000 research

Both 0.1% and 0.01% fipronil baits gave adequate control of wasps, However, it was concluded that the concentration of the active ingredient in 0.001% fipronil baits, was too low and did not perform as effectively as the higher concentrations. Freeze dried kangaroo mince when reconstituted with fipronil was also found to give similar control to equivalent concentrations of fipronil in fresh kangaroo mince bait, indicating that the freeze drying process may be a solution to reducing the perishability of a commercial bait product.

During 2000, it was also decided to enter discussions with Aventis CropScience, (the company holding the proprietary rights for fipronil), to encourage them to pursue registration of a commercial wasp bait containing fipronil.

2001 SEASON

Two series of trials to assess the efficacy of fipronil in baits were performed at several sites across metropolitan Adelaide. The first series of trials focussed on reducing the number of steps (days) required for successful baiting while the second series looked at minced beef as an alternative to fresh kangaroo matrix for broad area baiting.

Discussions with Aventis CropScience indicated that, given the promising outcome of studies conducted by SARDI in Adelaide and Landcare Research in New Zealand in 1999 and 2000, Aventis were keen to pursue the development and commercialisation of fipronil for baiting vespid wasps in a global market. Aventis CropScience also indicated that they did not wish to proceed further with kangaroo meat as a bait. Cultural sensitivities in some countries associated with the harvesting of kangaroos made this meat inappropriate for an international market. In late 2000, Aventis CropScience contracted both research groups (SA and NZ) to conduct further baiting research in the 2001 wasp season (February to May) with a view to select a non perishable protein which was attractive and acceptable to wasps and suitable for global use.

Materials and methods

Instantaneous chemical baiting trials using fresh kangaroo meat

Fresh minced kangaroo meat incorporated with 0.1% and 0.01% fipronil was tested at 4 and 2 sites respectively. Six bait dispensers were used at each trial site but the pre-baiting step used in all previous studies was eliminated from the methodology to determine if this step was essential for baiting success. These studies were conducted in the general area where the nest location was known and the nest traffic rate was monitored before and after toxic baiting to provide evidence of baiting efficacy.

Baiting trials using fresh minced beef with pre-baiting.

Trials using 0.1% fipronil with fresh minced beef as the bait matrix were conducted at 4 sites using the same broad-area methods as previously used included a pre-baiting step.

Results

Instantaneous chemical baiting trials using fresh kangaroo meat

Fipronil at 0.1% concentration reduced the numbers of wasp foraging to zero on bait stations at three of the four sites within 24 hours, and to zero at all four sites within two weeks (Table 1). All source nests monitored also collapsed within two weeks of the toxic-bait placement. Similar results were obtained at the two trial sites using fipronil at 0.01% concentration. Within 24 hours wasp traffic at one site was reduced to zero however, at the second site to less than one wasp per bait dispenser.

Chemical baiting trials using fresh minced beef with pre-baiting.

Beef mince with 0.1% fipronil suppressed foraging wasps totally within 24 hours at three of the four sites, with less than one wasp per dispenser remaining at the fourth site. All sites were free of wasps within two weeks of toxic baiting.

Table 1 Summary of trials using 0.1% fipronil in fresh kangaroo meat with no pre-baiting showing mean number of wasps (±SD) on bait stations and corresponding nest traffic counts (wasps/min)

			Mean number of wasps/bait(±SD) (n=6)		Post treatment	Nest Counts	
Site	Site	Date	0.1% Toxic bait	24hr post treatment	(2 weeks later)	Prebait	24hr Post-bait
1	Kurralta Pk	18/01/01	3.50±1.87	2.5±6.12	Nil	14.2±5.12	0
2	Torrensville	18/01/01	2.17±1.47	0	Nil	51.6±4.28	10.2±6.10
3	Unley	24/01/01	0.60±0.89	0	0	62.8±6.53	1.60±1.52
4	Unley	24/01/01	2.17±1.94	0	0	N/A	N/A

Table 2 Summary of trials using 0.01% fipronil in fresh kangaroo meat with no pre-baiting showing mean number of wasps (±SD) on bait stations and corresponding nest traffic counts (wasps/min)

			Mean number of wasps/bait(±SD) (n=6)		Post treatment	Nest Counts	
Site	Site	Date	0.01% Toxic bait	24hr post treatment	(2 weeks later)	Prebait	24hr Post-bait
1	Glenelg	30/01/01	1.00±0.89	0.17±0.41	0	41.2±5.07	3.20±1.64
2	Malvern	7/02/01	6.67±8.26	0	nil	N/A	N/A

Outcomes from 2001 research

Trials conducted during this season without the pre-baiting step indicated that this step was not essential for effective wasp baiting at sites where moderate to high numbers of wasps were foraging. However, it was recognised that a threshold exists for foraging wasp pressure below which the method will not give satisfactory results. The results from trials using mince beef baits suggested that beef may be a suitable bait substrate to replace kangaroo mince.

Aventis CropScience in the last two years have extensively supported the thrust of the South Australian project to produce a commercial wasp bait for the market in Australia. However, the contracts between Aventis CropScience with SARDI and Landcare NZ were commercial-in-confidence and hence only a broad overview of results are presented in this report. It was recognised by Aventis CropScience that a canned product would be easier and quicker to commercialise than pursuing the freeze-dried product approach. Acceptable proteins for canning were identified and combined with fipronil. These baits were produced in NZ and packaged in steel cans which provided a solution to the storage issues of perishable baits. Excellent wasp control was achieved with the first batch of canned baits in both countries. At the conclusion of this work, the shelf life of these canned products still remained to be assessed.

2002 SEASON

SARDI undertook a second series of trials for Aventis CropScience during this season. These trials were aimed at assessing the efficacy of three different new batches of canned products provided by Aventis CropScience. In addition, two batches of cans that were produced and canned 12 months earlier were assessed to test the shelf life of these products. Both of the latter batches were tested during the 2001 season shortly after the canning process and were found to be highly effective in controlling wasp populations. Further assessment of the need for the pre-baiting step and the optimum number of bait dispensers required per baiting site were also included within these trials.

Detailed results from the Aventis CropScience trials were again confidential, however, overall the baiting was significantly less successful compared to the previous season. Aventis CropScience have now identified technical problems with the canning process used for the new bait formulations tested and with the shelf life of the baits canned for the 2001 season. This has temporarily set back the commercialisation process of their new wasp bait and it is now unlikely that a new product will be available "off-the-shelf" in the immediate future. The efforts to assess the need for a pre-baiting step and determine the optimum number of bait dispensers per site were confounded by these variable results and further research is necessary to solve these problems.

Recent discussions (September 2002) with Aventis CropScience (recently absorbed by Bayer Ltd) have revealed that they are currently making their strategic plan aiming at producing a global wasp bait. Given the delays in non perishable bait production due to technical difficulties, SARDI believes there is an opportunity to fill the gap in availability of a fully commercial bait product. Fipronil (0.1%) incorporated in kangaroo mince bait and presented to wasps in appropriate dispensers may be used under a permit which issued by the National Registration Authority in Australia. Once registered, there is a possibility for controlled use of the fresh meat toxic baiting technology in South Australia in the coming wasp season in early 2003.

PATENT

- A toxic bait dispenser differs from a bait trap in that it allow wasps to remove bait and return to the nest. The dispenser also houses the bait so that domestic pets and native vertebrates are protected from access to the toxic bait. SARDI has recognised that an importance of safe environmental practice and has developed a functional prototype bait dispenser. The intellectual property for the bait dispenser for Project 1 was initially protected by SARDI filing for a provisional patent.
- Australian Provisional Patent Application No PR1849 filed December 1st 2000

This progressed to an application for a full patent earlier this year.

- Patent Cooperation Treaty Application No. PCT/AU01/01564 An apparatus and method for controlling wasps or yellow jackets. Minister for Primary Industries and Resources, June 2002.

A recent examiner's report for the full patent concluded that 9 of the 10 claims made in the patent are not new and inventive largely because of a similar patent lodged in the USA. This report is currently being considered by the Office of Local Government and it seems likely that the application for full patent will be withdrawn.

DISCUSSION

From the data generated over the last four wasp seasons, SARDI is confident that wasp baiting has a role to play in the future management of wasp problems in southern Australia. Until a fully commercial product is available to the market there is convincing evidence that, 0.1% baits containing fipronil in fresh kangaroo mince will give excellent control of nuisance populations of wasps and associated nests. It is stressed that this baiting technology will only be effective where there are sufficient numbers of foraging wasps present. Too few wasps present will result in insufficient quantities of bait being transported back to the nest and colony destruction may not occur. It is also recognised that where the nest location is known, direct treatment of the nest with pesticide remains the most effective way of dealing with the wasp colony. The baiting

technique should focus on problem areas where the nest is difficult to access or the location is not known. The controlled use of fresh baits under this type of permit will increase familiarity with toxic baiting systems and has the potential to enhance the adoption of any commercial product when it is released.

To minimise any possibility of off-target effects, toxic baits should be presented in an appropriate bait dispenser such as those used in the SARDI trials over the last four seasons. During this time, the bait dispenser successfully excluded all vertebrates (birds, lizards, domestic pets, etc) from the baits and few invertebrate species (pest flies such as blowflies and flesh-flies) other than European wasp were affected by the baits. Honey bees and native wasps were not attracted to the baits. The prototype dispenser used in SARDI's research studies would be adequate for dispensing toxic wasp baits under an NRA permit. Once a fully commercial non-perishable bait is registered for the market, it is likely the final product would need a bait dispenser to be included as part of the commercial package.

RECOMMENDATION

It is recommended that, in addition to direct nest destruction Local Government bodies in South Australia should investigate the feasibility of using baiting technology under an NRA permit as a management strategy against European wasp until a commercial bait product is available.

MEDIA and PRESENTATIONS

European wasp Collaborative Project Meeting - September 1999, CSIRO Entomology, *Chemical Baiting* - Dennis Hopkins
European wasp Collaborative Project Meeting - March 2000, An overview of the SARDI baiting project and results was presented to the stakeholders. It is anticipated that there will be a presentation of our results to date for the Pilot Research Councils who were involved in the experiments this year.
European wasp Collaborative Project Meeting -December 2000**.** Presentation at meeting in Tasmania
West Torrens Council - December1999, presentation on *The biology and control of the European wasp*, Glenys M Wood and Andrew F Reeson
Ecology Society of Australia - April 2000, Waite Campus
Toxic baits for European wasps - a free lunch with a sting? - Glenys M Wood
National Science Week - 2000 Waite Campus, Workshop on European wasp biology for high school students, Glenys M Wood
National Science Week 2001 involving European wasps within the University Entomology Media/Extension, interactive displays
Australian Viticulture 2002 *Baiting of European wasps is refined* Vol 6: No 2
Contributions to Media, local government news and newsletters
Wasp Times - December 1999- *Toxic Baiting in South Australia or "There is no such thing as a free lunch"* - Glenys M Wood
The Helix - **No. 68** October/November 1999. *Taking advantage of European wasp loyalty* Sarah R Phillips
Meadows Rural Watch Newsletter- *European wasp report*
City of West Torrens 'Talking points' *European wasps 'A Case Study'*

Advertiser and **Sunday Mail** - collaboration on several articles written throughout active part of the wasp season.

- Rex Jory, October 1999 *Be told: watch for wasps*
- Ben Hopper, January 2000 *Ground gained in war on European wasps*
- David Nankervis, February 2000 *Wasp Invaders on the rise*
- David Nankervis, March 2000 *Fight to keep wasp numbers from soaring*
- Jemma Chapman, December 2001 *The 500,000 killer wasps that lived in a suburban backyard*

The Courier, Mount Barker -

- December 1999 *Toxic tests on wasps*
- March 2000 *Rise in wasp nest eradication*
- April 2002 *Wasp numbers up*

Hills and Valley Messenger

- October 2001 *European wasps: Numbers may rise after 4-year drop: expert*

Adelaide Hills Weekender

- January 2000 *European Wasp research effort*

Television –

- January 2000 News coverage with Channel 10.
- Channel 10 pre budget coverage on wasp funding for the coming year May2001

Radio - Interviews- Dennis Hopkins and Glenys Wood

CONFERENCES

Australian Entomological Society 30th AGM and Scientific Conference, September 1999. The Australian National University, Canberra,
The impact of toxic baiting on the urban European wasp Vespula germanica Glenys M Wood and Dennis C Hopkins

Australian Entomological Society 31th AGM and Scientific Conference June 2000. Northern Territory University, Darwin.
New initiatives toward the control of a social insect pest Vespula germanica Glenys M Wood and Andrew F Reeson

Ecological Society of Australia Conference Poster presentation November 2000, *Control of the European wasp Vespula germanica in urban South Australia* A F Reeson, G M Wood, D C Hopkins and A D Austin

WEB site development – SARDI now has a wasp facts and identification web page

http://www.sardi.sa.gov.au/entomology/index.html

ACKNOWLEDGMENTS

Pilot Research councils provided assistance in finding experimental sites, arranging access to landowners properties and gave other support. ForestrySA rangers from Kuitpo and Mt Crawford assisted with finding wasp sites and provided support and transport for baiting trials where necessary.

We would also like to acknowledge financial support from Bayer Australia Ltd (formerly Aventis CropScience).

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PROJECT 2 – WASP ECOLOGY/POPULATION MODELLING

PhD Student:	Marta Kasper
Honours Student:	Kym Perry
Project Supervisors:	Professor A. D. Austin
	Dr. D. Mackay, Flinders University
	Dr. S. Cooper, SA Museum
International Collaborators:	Professor R. Matthews, University of Georgia
	Dr Jacqueline Beggs, Landcare Research Ltd, New Zealand
	Dr Richard Harris, Landcare Research Ltd, New Zealand
Commencement date:	April 1999
Completion date:	January 2003

NB. Due to the late start of the PhD student, Ms Kasper, this project will continue until early 2003

AIMS

The three main aims of this project were:

1) to study the basic biology and ecology of the European wasp in South Australia;
2) to determine the factors influencing wasp population size, including climatic data, foraging conditions, quality of new queens, and previous numbers; and
3) to use this information to develop a model predicting wasp populations in South Australia from year to year.

BIOLOGY AND ECOLOGY

Nesting Preferences

In September 1999 eight metropolitan councils chose to participate in this project by recording the locations of all nests they destroyed on a standard form. Not all of the promised data was forthcoming, and in 2000/2001 only four council participated, while in 2001/2002 this was reduced to one. For this reason, only data for the first two seasons is presented here. The overall trend is clear in that more nests are constructed in buildings and aerial locations compared with the ground (Figure 1). However, there is also significant variation from season to season in the relative proportion of nest locations, and this may be due to early warm spring weather followed by prolonged rains at the beginning of summer.

Colony Development

Wasp colony development during the season was monitored over three seasons, with over 60 nests from six council areas being collected. These were anaesthetised prior to excavating, then frozen to kill the wasps inside. The nests were then examined for the number of layers, small and large cells, generations reared in each cell, eggs in cells, and the sexes of capped pupae. The data are similar for the three seasons but, again, show variation in the length of the season, with the 1999/2000 season being the longest. In 1999 nest initiation occurred in October/November (Figure 2) but later in 2000 and 2001 (December), and continued longer, until May 2000 versus April in 2001/2002. These results will be used

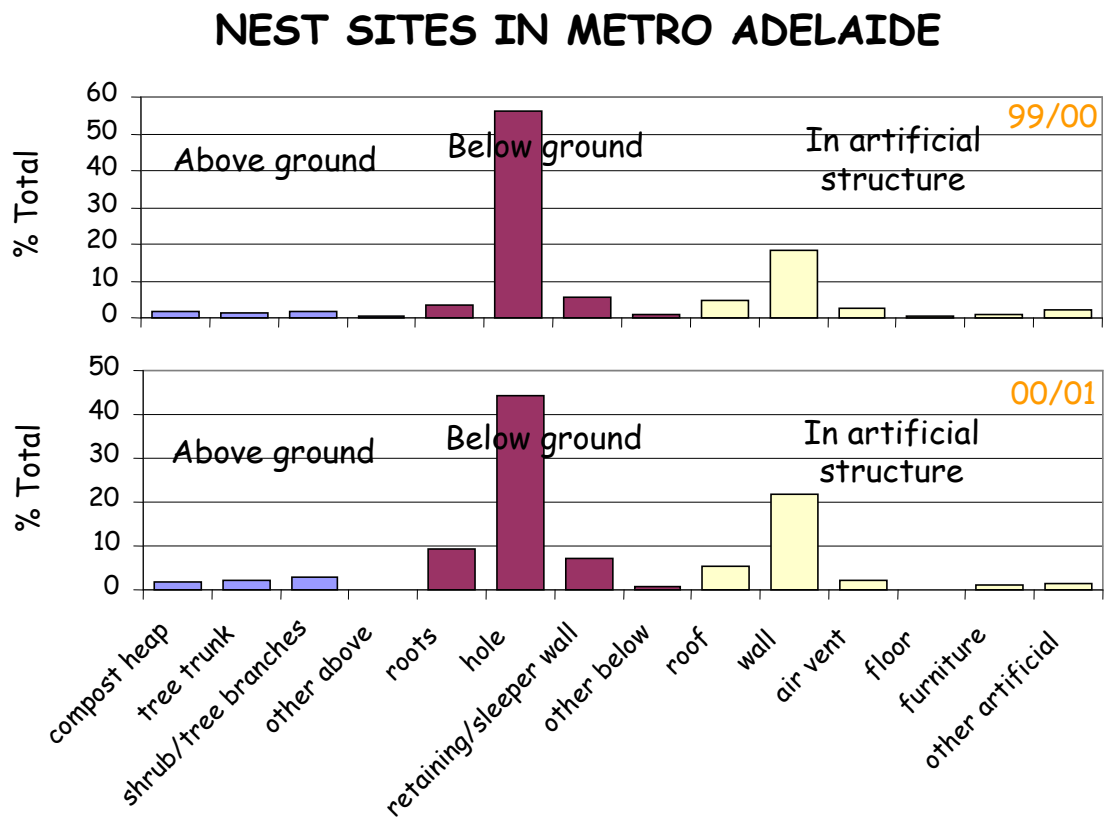

Figure 1: Proportion of European wasp nests found in various locations in metropolitan Adelaide over two seasons, 1999/2000 and 2000/2001.

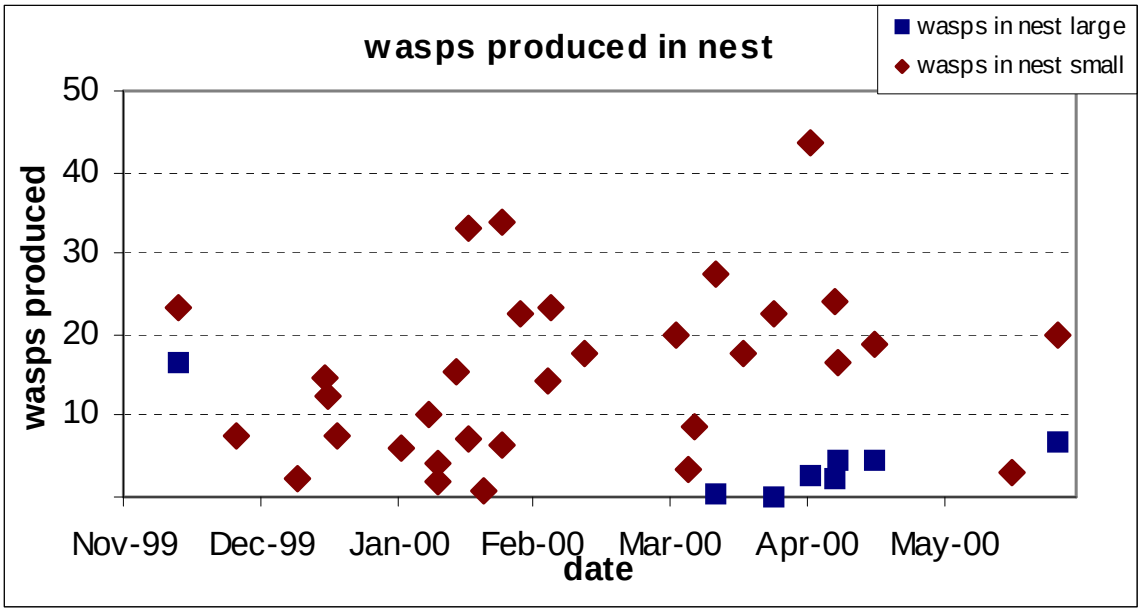

Figure 2: Example of data obtained from analysing nests for numbers of wasps produced. N.B. Data for other seasons is very similar.

to provide critical information for constructing a model of wasp colony growth in South Australia (this is currently underway and will be completed in early 2003 as part of Ms Kasper's PhD thesis - see below).

Daily Foraging Activity

Wasp traffic rate was measured as the number of wasps flying in and out of a nest per unit time, and was the only viable external measure of nest size and activity. Two variables have been identified which significantly influence traffic rate - these are light intensity and temperature.

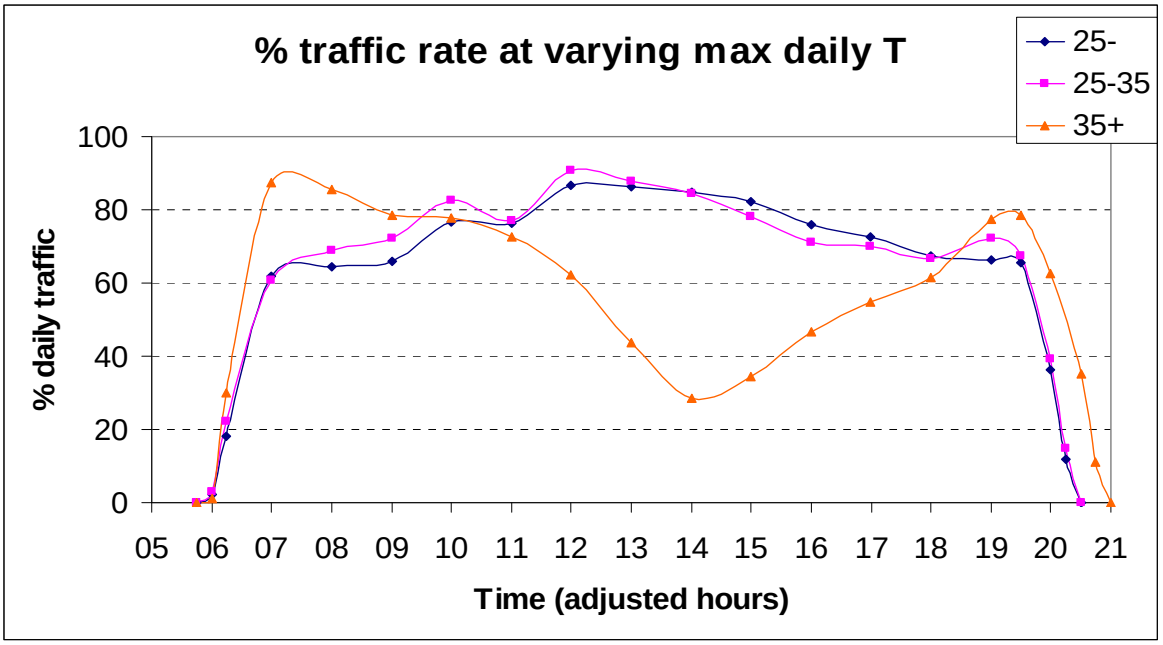

Figure 3: European wasp traffic rates, expressed as % maximum daily per nest, adjusted for seasonal variation in day length. Shown are averages for days when the maximum air temperature was below 25°C, between 25.5 and 34.5°C, and 35°C and above.

Over 55 observations of 15 nests were undertaken over a three-month period (January-March) under different conditions to determine the effect of these variables. Traffic rate (wasps entering/exiting per minute) measured throughout the day shows that wasps start foraging as soon as there is light, rapidly increase to a high foraging rate and continue at above 60% until just before dark, when activity ceases abruptly (Figure 3).

Most deviations from this pattern were related to temperature. Wasp activity decreased significantly on days when the maximum air temperature was above 35°C. Temperatures below 10°C also retarded wasp activity (Figure 4), however this occurred infrequently during summer and early autumn in Adelaide when average day time temperatures were usually well above this. Other factors influencing activity levels were heavy or prolonged rain, and fog. Humidity levels did not affect traffic rates (data not shown here).

The data have been modeled as a function of temperature and light intensity, and a predictive output of wasp traffic during the season is currently under development and will be included in Ms. Kasper's PhD thesis.

Prey Items

In order to assess whether food is a limiting factor in the distribution of wasps, and also to study the impact of wasps on native invertebrates, food items brought back to the nest by workers were sampled from different locations and at various times during the season. Over the past two years (2000/2001 and 2001/2002), 744 items were collected from 4360 foragers

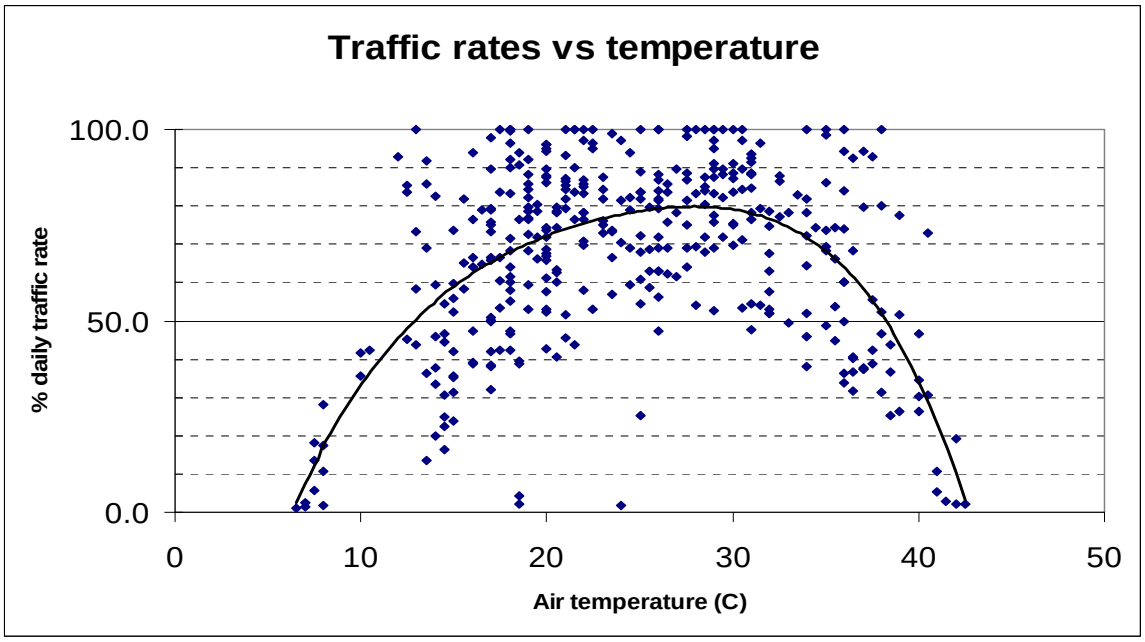

Figure 4: European wasp traffic rates, expressed as % maximum daily per nest, at varying air temperatures. Each point represents the average of 10 1-minute counts of wasps entering or leaving the nest.

(Figure 3). The items most frequently brought back to the nest were Diptera (flies, 35%), followed by Hymenoptera (mostly *Apis mellifera*, honey bees, 9%), Lepidoptera (caterpillars, 4%) and Arachnida (spiders, 3%). Other prey items included grasshoppers and crickets, amphipods, beetles, lacewings, leafhoppers, dragonflies, as well as other unidentified items. Twenty-two percent of all items were so badly masticated that they could not be identified visually. Current work (to be completed in late 2002) is aimed at developing molecular methods to identify these items, so as to eliminate any biases in the data. Wood pulp, which the wasps use in building and expanding their nests, accounted for 19% of all loads brought back to the nest.

The above data support observations from Europe and New Zealand, that *V. germanica* is a generalist forager and that it probably responds opportunistically to specific locally abundant prey. This would explain the difference between the data presented in Figure 5 and that recorded in New Zealand where spiders and lepidopteran caterpillars comprised a relatively higher proportion of total prey collected. Given that wasps are generalists, it is unlikely that they are, overall, having a detrimental effect on native insect populations. However, in seasons when wasps populations are extremely high, they may cause a reduction in total insect diversity and abundance, although further research would be required to determine this conclusively.

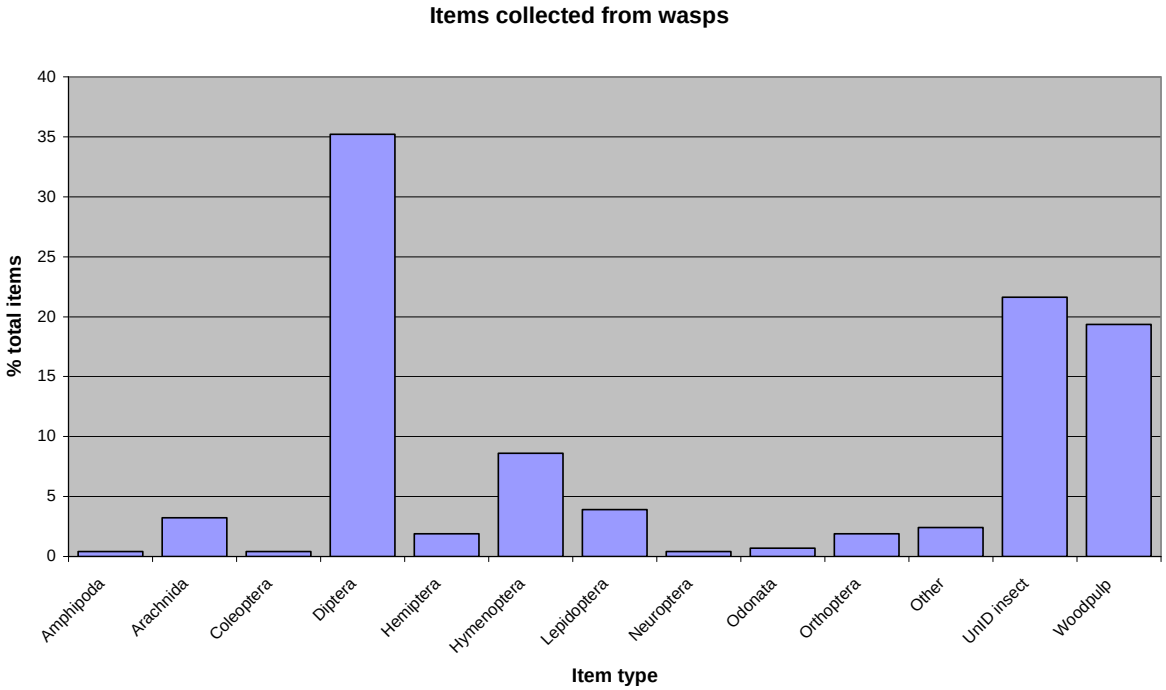

Figure 5: Items, expressed as %total, collected from European wasp foragers returning to the nest (2000/2001 and 2001/2002 combined)

Comparison of *Vespula* and *Polistes*

To determine whether *V. germanica* adversely affects populations of the native social wasp *Polistes humilis*, the honours student Kym Perry, undertook a project in 2001 to investigate whether any competition for resources occurs between these two species. In brief, *P. humilis* mostly forages for prey in different microhabitats, and it specialises in collecting lepidopteran larvae (100% of its protein intake versus 4% for *V. germanica*). Foraging behaviour is also different for *P. humilis* in that it does not fly from the nest at temperatures below 15^{o}C, but is capable of foraging at much higher temperatures than *V. germanica* (>40 ^{o}C). Finally, the size and location of *P. humilis* nests are different than those of *Vespula*. They are mostly constructed in exposed situations (in vegetation or under the eaves of buildings), and are rarely larger than 100 individuals.

Over-wintering Queens

One of the major factors postulated to regulate the size of wasp populations, at least early in the season, is differential mortality of hibernating queens. An experiment was undertaken during 2001 to determine the length of hibernation and weight of queen wasps. Hibernating queens were placed into wooden chambers to simulate their natural winter positions, and placed at varying temperatures, both in controlled temperature cabinets and in the field. Results show that initial queen weight determines the duration of hibernation (Figure 6). Hibernating for longer periods may ensure that queens start new nests at the most opportune time, and thus nests producing heaviest new queens at the end of the season may form more nests the following season.

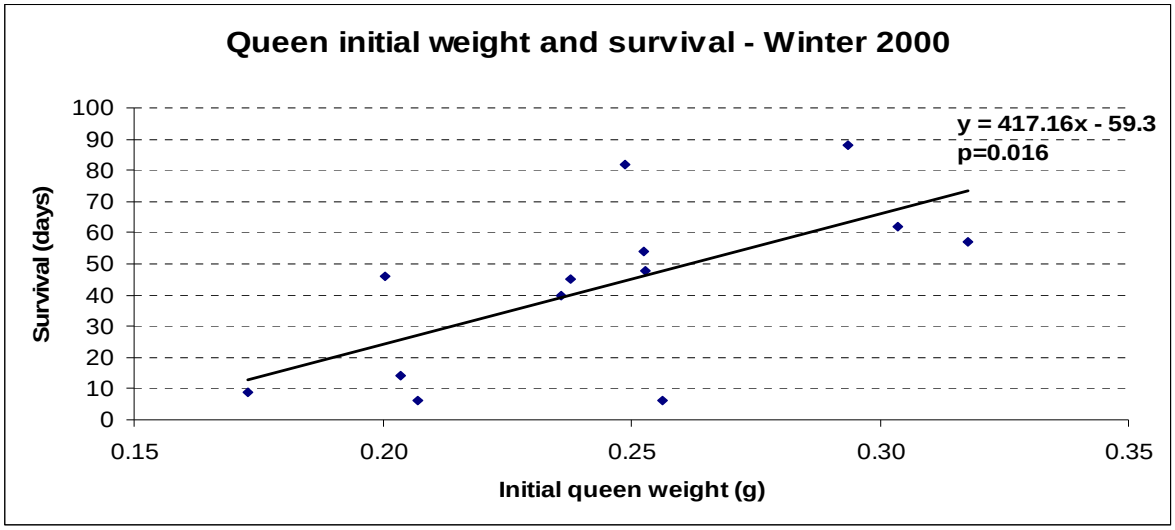

Figure 6: Initial weight of new queens subjected to hibernation and their survival, in days from starting the experiment.

POPULATION GENETICS

The aim of this component of the project is to use molecular techniques to examine the relatedness of wasp nests within the Adelaide region to determine their likely source of origin and thus, degree of dispersal. This should prove useful in determining the mode of spread of wasps, ie. does it occur on a local scale, or are queens dispersing over longer distances using human mediation? In order to achieve this, four microsatellite loci are being screened for variation. This part of the project is still underway, will be completed by early 2003, and will be included in Ms. Kasper's PhD thesis.

DEVELOPMENT OF A POPULATION MODEL

One of the major aims of this project is to develop a population model that can be used to predict potential population size in the next season. Most of the relevant information is now available to do this (see above), and preliminary work is now underway to develop the model. This is being undertaken by Ms Kasper and Dr Mackay (Flinders University) and is scheduled to be completed early in 2003.

DIFFICULTIES ENCOUNTERED

The input of the LGA into the project has decreased substantially over the three years, with no input at all in the past year. Also, while some councils have been extremely cooperative, others have had less involvement. This has caused some difficulties in obtaining information about nest locations – for example, only one of the 15 nests used in the traffic rate monitoring was reported by a council. As already mentioned under "Nesting Preferences", data on wasp nest location was only collected by one council during the final season. Also, obtaining geographic data on the location of wasp nests destroyed (i.e. street addresses; a crucial part of predicting population size) has become more difficult, as it was necessary for councils to submit these to the LGA with their rebate claims until 1999, but since then councils have to be contacted individually.

COMMUNICATION &CONFERENCES

During the three years of the project Professor Austin, Ms Kasper and others have disseminated results of this research through numerous radio interviews, television appearances, assistance in the preparation of newspaper articles (for the *Advertiser, Australian* and *Mt Barker Courier*), the running of annual National Wasp Workshops, presentations to the Local Government Association, local Councils and to various public interest groups, formal presentation of results at national and international conferences, and the development of a website (http://www.waite.adelaide.edu.au/europeanwasp).

Conference Presentations

2002 "The status of *Vespula germanica* as an urban pest in Australasia" (A.D. Austin, M. Kasper & A. Reeson) *14th International Congress of IUSSI, Sapporo, Japan*.

2002 "Population ecology of an invasive wasp *Vespula germanica* in South Australia" (M. Kasper, A.D. Austin, A. Reeson & D.A. Mackay) *14th International Congress of IUSSI, Sapporo, Japan*.

2001 "Prey overlap in a native and an introduced social wasp" (Poster: M. Kasper, K. Perry, A.F. Reeson & A.D. Austin) *5th Invertebrate Biodiversity & Conservation Conference, Adelaide.*

2001 "Daily activity at the nest entrance of a social wasp, *Vespula germanica*" (M. Kasper, & A.D Austin) *32nd Australian Entomological Society Conference, Sydney.*

2000 "Population ecology of an invasive social insect, *Vespula germanica* (Hymenoptera: Vespidae) in South Australia" (M. Kasper, A.D. Austin & D. Mackay*) Ecology in a Rapidly Changing World. Ecological Society of Australia Conference, Melbourne.*

Publications

2001 K. Perry, Honours Thesis, The University of Adelaide "Comparative feeding biology of the introduced *Vespula* and native *Polistes* wasps".

2000 R.W. Matthew, M.A.D. Goodisman, A.D. Austin & R. Bashford "The introduced English wasp *Vespula vulgaris* (L.) (Hymenoptera: Vespidae) newly recorded invading native forests in Tasmania". *Australian Journal of Entomology* 39: 177-179.

N.B. Several publications will be forthcoming from Ms Kasper's PhD thesis and these will be submitted to refereed scientific journals in 2003.

ACKNOWLEDGMENTS

We wish to thank the following people for their help and assistance in various ways with Project 2:

- Allin Hudson, Urrbrae Wetlands
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- Nerylee Watson, City of Mitcham Council
- Nick Stevens, University of Adelaide
- Nicki Featherstone, University of Adelaide
- Prof. Otto Schmidt, University of Adelaide
- Quentin Schulz, City of Tea Tree Gully Council
- Richard Glatz, University of Adelaide
- Rick Vetter, University of California, Riverside
- Rob Vickers, PIRSA, Lenswood
- Sassan Agsari, University of Adelaide
- Travis Gotch, University of Adelaide
- Trevor Dix, Adelaide Hills Council

PROJECT 3 - BIOLOGICAL CONTROL INITIATIVE

Postdoctoral Fellow:	Dr A. F. Reeson
Project supervisor:	Professor A.D. Austin
International Collaborators:	Dr Jacqueline Beggs, Landcare Research Ltd, New Zealand Dr Richard Harris, Landcare Research Ltd, New Zealand Dr Travis Glare, AgResearch Ltd, New Zealand Professor R. Matthews, University of Georgia
Commencement date:	July 1999
Completion date:	July 2002

AIMS

This project had three main aims:

1) To undertake a detailed review of the literature on potential long-term biological control options for European wasp;
2) Using information available from the literature and preliminary results from Project 2, determine the most likely strategy in the Adelaide region; and
3) Undertake a detailed survey of diseases and natural enemies of native wasps and their efficacy against European wasp.

ASSESSMENT OF BIOLOGICAL CONTROL OPTIONS

Parasitoids

The first and second of the above aims were largely completed within the first year of the project. Based on research world-wide and particularly that by our collaborators in New Zealand, a number of factors make *Vespula* a poor candidate for classical biological control. These can be best summarised as follows:

- Analysis of the literature and discussion with Drs Beggs and Harris strongly suggest that the parasitic wasp *Sphecophaga vesparum* (Ichneumonidae), is not an effective biological control agent in New Zealand and earlier establishment attempts in Australia had failed.
- The life history characteristic of having a long generation time and its inability to access most larval cells within a nest mean that *S. vesparum* can only parasitise a small proportion of any wasp generation. Further, population modeling in New Zealand has indicated only a 10% reduction in wasp densities.
- Other species of parasitic Hymenoptera recorded from vespids around the world are either polyphagous and could shift onto non-target hosts if introduced into Australia, or show a relatively high degree of host specificity and are unlikely to be useful against *Vespula*.

For the above reasons classical biological control using parasitoids was not considered as a viable option in South Australia.

Microbial Pathogens
The second two years of the project comprised a detailed survey of pathogens associated with wasp nests, larvae and adults in South Australia employing bioassay, microscopic and molecular techniques, and assessment of their potential as microbial control agents. In addition, a number of pathogens used against other insect pests were tested in conjunction with colleagues in New Zealand. A summary of results is presented below.

Protozoa
A microsporidian pathogen, originally identified as *Nosema* sp. and isolated from diseased *Vespula* by CSIRO Entomology, was assessed in detail to determine its efficacy against wasps nests. Material supplied by CSIRO was tested in preliminary laboratory trials against wasp larvae and proved ineffective. Follow up research by Dr Dennis Anderson (CSIRO) determined that the species involved belonged to the genus *Vairimorpha* and sequence data showed it to be closest to a pathogen previously isolated from the lepidopteran *Lymantria dispar*. Further, while our experiments indicated that it does not pose a threat to *Apis mellifera* (honey bees), *Vairimorpha* spp. are known to have a very broad host range, and therefore are generally unsuitable candidates as biological control agents.

For these reasons,* Vairimorpha *was unlikely to have potential as a control agent and work on it was discontinued.

Bacteria Isolated from Nests
Bacteria from nests of both *Vespula germanica* and the native vespid *Polistes humilis* have been found to be pathogenic towards *V. germanica* larvae. Work to identify these bacteria has employed cloning a highly conserved gene (16S rRNA), sequencing it and comparing the sequence against the Genebank database. This method has the advantage of accurately identifying known bacteria while at the same time indicating the closest known relatives of undescribed bacteria. Furthermore, it does not require that the isolate be first cultured. A summary of our assessment of the bacteria recorded from wasps in South Australia follows:

1) An unknown species close to *Providencia stuartii* is a strain is a proteobacteria. Its closest relative is an undescribed bacterium recorded in GenBank that was isolated from a fruitfly *Drosophila paulistorum*, but is only known to be pathogenic against Lepidoptera.

2) *Serratia marescens*. This is common soil organism and known pathogen of insects, which caused high levels of mortality in bioassays. However, it has been known to infect humans and therefore is not useful as a biological control agent.

3) An undescribed taxon close to *Lactococcus lactis*. This strain was collected from two separate nests, and is part of a group of bacteria that ferment carbohydrates to produce lactic acid. They are found in nutrient rich environments, including the intestinal tracts of animals. While the strain isolated here can be pathogenic, most likely it can only invade host insects that are already weakened by some other agent.

4) *Proteus mirabilis*. This is another common bacterium of soil and water, which can be pathogenic under certain circumstances.

5) Spore-forming bacteria: *Bacillus thuringiensis, B. pumilis* and *B. mycoides/ weihenstephanensis* were identified microscopically from wasp cadavers, and several isolates successfully cultured. Spores of these bacteria enable them to survive in the

environment, protecting them from extremes in temperature and desiccation, until they encounter an insect host. Many strains of *Bacillus* are widely used in biological control so their occurrence in wasps was encouraging. However, they were not considered as likely candidates for effective biological control because their pathology is similar to spore-forming fungi which have been shown by our New Zealand colleagues to be of only limited application (see below).

Pathogenic Fungi

A great number of fungi have been isolated from wasp nests in Australia and New Zealand although most are probably saprophytic, i.e. growing on disgarded material within nests. Entomophagous forms such as *Aspergillus flavus* and *Beauveria bassiana* are also known from nests, although these may be associated with prey brought into the nest rather than with wasps themselves. Extensive laboratory bioassays undertaken in New Zealand have shown *A. flavus*, *Beauveria bassiana* and *Metarhizium anisopliae* can be pathogenic against wasps. *Beauveria bassiana* has also been shown to be effective against nests when workers were sprayed. However, this was only achieved with very intensive application and only appeared to be effective in a limited area under humid conditions. Two factors drastically restrict the potential of fungi as biological control agents.

- Application of fungal spores under field conditions in Australia is very likely to be ineffective as the microclimate around nests is very dry and will inhibit the development of fungal epizootics.
- Extreme hygiene behaviour exhibited by workers in the nest (see below), means that initial infections are recognised and diseased larvae removed from the nest before spores form and have a chance to spread through the nest. This behaviour therefore provides a barrier against horizontal transmission of disease within the nest

Although further assessment of some bacteria and fungi might be worth pursuing in the future, their immediate potential as control agents appears to be restricted compared with other candidates.

Nematodes

Entomopathogenic nematodes are used against a wide range of insect pests, and were therefore considered as a possible control agent for *Vespula*. There is some evidence that *Steinernema carpocapsae* is active against a number of wasp species, although there is only one record of a naturally occurring infection. Mortality is caused by symbiotic bacteria rather than the nematodes themselves; the role of the nematode is to penetrate the insect haemocoel, where it releases the symbionts (*Xenorhabdus* spp. bacteria) which kill the host and create conditions which facilitate nematode reproduction (see review of the literature presented in the 2001 Annual Report).

Nematode Field Trials

To determine the effectiveness of nematodes as potential biocontrol agents for wasps, field trials were undertaken in New Zealand in conjunction with Dr Richard Harris, as the species of nematodes to be assessed already existed there. This work would have been difficult to carry out in Australia because of quarantine restrictions. Two strains of nematode were used, *Steinernema carpocapsae* strain 'Heidi' and a second species known to be pathogenic against insects, *Heterorhabditis zealandica* strain 'Riwaka'. Field trials were carried out in the Pelorus Bridge scenic reserve, in the Marlborough region of New Zealand in February-March 2001. Native forests in this area have extremely high densities of introduced wasps

Methods: *Vespula vulgaris* nests were exposed to nematodes that were mixed in with a sardine bait. Laboratory studies confirmed that the nematodes were not inactivated as a result of being mixed in with the sardine baits. These baits were then placed immediately outside the nest entrance, where they were rapidly discovered by foraging workers. The baits consisted of approximately 40g of tinned sardines mixed with 2ml of 3600 nematode/ml suspension. Nests were treated with baits containing either *S. carpocapsae*, *H. zealandica* or water as a control. Nest activity was then measured by recording the traffic rate of workers entering and exiting the nest over four consecutive one minute periods. Traffic rate was recorded at the same time each day for five days following the introduction of the baits. On the sixth day approximately half of the treated nests were destructively sampled in order to look for any signs of infection within the combs.

A second field trial was carried out to determine whether infections were established in the nest immediately after the introduction of nematodes. *Vespula vulgaris* nests were treated with sardine baits containing 2ml of 9000 nematodes/ml suspension of *H. zealandica*. Nests were destructively sampled at one, two and four days after exposure to nematodes and examined for signs of infection. Combs from these nests were cleared of workers and taken back to the laboratory where they were monitored for several days to check for any nematode-induced larval mortality.

Results: In the laboratory bioassays, *H. zealandica* caused 40% larval mortality at the high dose and 5% mortality at the low dose. There was no larval mortality associated with exposure to *S. carpocapsae*. No nematode-associated mortality was seen in the worker bioassays; workers in both the treatment and control groups died within three days. Exposure to nematodes did not affect nest activity; there was no significant difference in activity rate between untreated control nests and those treated with either nematode on day four (ANOVA, $F=0.4$; $p>0.05$) nor on any previous day. When the nests were destructively sampled after six days no sick or dead larvae were observed. Two of the nests treated with *H. zealandica* contained no mature larvae, although eggs and pupae were present. However, due to the wide variation in developmental stage among nests it was not possible to demonstrate any consistent differences between treatment groups. In the second field trial, no nematode-induced larval mortality was observed in any of the nests.

Conclusions: The strain of *Steinernema carpocapsae* used in these experiments was not capable of killing wasp larvae. Other authors have found *S. carpocapsae* pathogenic against *V. germanica* larvae and workers, although these studies used a different strain of nematode. *Heterorhabditis zealandica* was shown to be capable of killing wasp larvae. However, despite promising results from laboratory assays, these field trials suggest that nematodes do not have potential as biocontrol agents for wasps. There was no indication of nests being successfully infected in the field. This is surprising as the baits contained huge numbers of nematodes that were known to be infectious in the laboratory. The failure of the nematodes to infect nests in the field may result from the way in which the baits are processed by the wasps. The baits are chewed up by the foragers and manipulated into a small ball, which they carry back into the nest. The bait is then passed to other workers that chew it further before feeding it to the larvae, which then chew it themselves before ingesting it. This chewing action may destroy nematodes and it is possible that, by repeatedly chewing the bait, the wasps inactivate the nematodes in it.

Alternatively, the microclimate inside nests may not favour nematode infection. The optimum temperature for most entomopathogenic nematodes is 20-25°C, with activity usually much reduced at 30°C. Previous research overseas suggests that the optimum temperature for *Vespula*

vulgaris nests is 32°C, which may prove too high for successful nematode infection. Therefore elevated temperatures within the nest may account for the differences in infectivity observed between the laboratory and the field. There are nematode strains that are effective at higher temperatures; *S. riobrave* has been shown to infect lepidopteran larvae from 22°C to 36°C, with the optimum varying from 25°C to 29°C depending on the host species. Therefore future trials using heat tolerant strains may be useful. Alternatively it may be possible to extend the thermal tolerance of existing strains by culturing them at high temperatures.

Future research on Nematodes

- Undertake bioassays to determine if mastication destroys nematodes passed between workers and larvae.
- Explore tolerance and pathogenicity of different nematode stains to temperatures above 30°C.
- Test methods for increasing the thermal tolerance of existing strains by culturing them at high temperatures.

Overall Assessment of Pathogenic Biocontrol

Field surveys over three wasp seasons, remarkably, have revealed very little evidence of disease in *Vespula* nests in the Adelaide region. This may be due to hygienic behaviour by wasps, which have been shown by Steven Hardcourt (PhD student, Lincoln University, New Zealand) to have 'antiseptic-like' saliva and venom which may act to keep internal surfaces in the nest largely free of pathogens. Also, this phenomenon, in conjunction with the fact that sick or dying nestmates are removed by worker wasps (Hardcourt personal communication), provides an extremely effective barrier against horizontal transmission of diseases within the nest.

While a number of potentially pathogenic bacteria have been found within nests, they have not proved capable of causing consistently high levels of mortality in bioassays. Further, there would seem little short-term gain in pursuing research on microsporidians, nematodes and viruses, even though some are potentially pathogenic against *Vespula*. **Unless some way can be found to circumvent hygienic behaviour by wasps, there would seem to be little advantage in continuing research examining the effect of various candidate pathogens** under laboratory conditions. The wasp research group in New Zealand is considering an examination of potential behavioral disrupters in the future that could be employed in conjunction with pathogens, but this will be a long-term endeavour.

The Wasp Gut as a Novel Source of Potential Pathogens

Introduction: A social lifestyle and broad diet mean that wasps are likely to be exposed to many different bacteria. Foraging wasps collect live insect prey, carrion, fruit, nectar, water and wood pulp that are brought back and distributed within the nest. Solid food items, including invertebrate prey and carrion, cannot be ingested by the workers and so are fed to the larvae, which in some ways act as the 'digestive system' of the colony. The larval gut is unusual compared with many non-insects in that waste is not egested until pupation, so substantial material builds up over the course of larval development. This unusual environment is likely to play host to many bacteria, some of which may play a role in digestion while others may be potential pathogens. We have examined these microbial communities to determine whether there is a certain bacterial profile associated with *Vespula* nests; such a profile would enable diseased nests to be rapidly identified as they will contain different bacteria. We also looked for evidence of symbiotic bacteria, i.e. bacteria that live in the wasp gut and play a role in digestion. In other species of social insect, symbiotic bacteria are essential for healthy functioning of the colony. So

the symbiotic bacteria may represent a target for control, one with fewer defenses than the wasps themselves.

Approach: Initially, two methods were considered to examine these bacterial communities. These were 1) plating out gut contents sampled from colonies on a variety of artificial media, growing colonies on these media , then subsequently isolating and identifying them; and 2) isolating DNA extracted from wasp guts, using PCR using primers for the 16S gene in all bacteria, and examining PCR products using a denaturing gradient gel electrophoresis (DGGE) system, which can separate DNA fragments from different sequences (i.e. bacterial species)
.
While the colony isolation method has identified a number of bacterial strains commonly found associated with wasps, including *Lactococcus*, *Providencia*, *Proteus* and *Bacillus*, **DGGE has proved to be a far more powerful tool for studying these communities**. The principal advantage of DGGE is that it is not constrained by the ability of bacteria to grow on artificial media. Most microbiological studies rely on getting bacteria to grow on artificial compounds such as agar. However, it is increasingly being realised that only a small proportion of bacteria can be grown under these conditions. Using DGGE we were able to search the whole of the bacterial spectrum for species of interest. These techniques were also applied to samples from the guts of native *Polistes* paper wasps.

Results: The results of this study have been submitted for publication to the prestigious journal *Insect Molecular Biology*, a copy of which is included here as Appendix 1. The results and outcomes of this work can be summarised as follows:

- There was considerable variation in the gut flora of larvae among nests from different areas of Adelaide and at different times of year.
- Some bacteria were present in most of the nests sampled, suggesting they may have an intimate association with wasps.
- One species has been identified as a new species, which is most closely related to *Rickettsiella grylli*, an insect pathogen.
- A number of strains of this pathogen have been identified from wasp nests.
- *Polistes* paper wasps were found to have far fewer species of bacteria in their guts. No pathogens were identified. One species appeared to be present in all *Polistes* samples; a similar species was widespread in *Vespula* nests.

Outcome: The *Rickettsiella* has been targeted by the New Zealand research group as a potential pathogen worthy of further investigation. In collaboration with Professor Austin and Dr Reeson, they have initiated a project to identify where it is located in the wasp gut, and to compare it to the occurrence and identity of *Rickettsiella* in New Zealand.

COMMUNICATION &CONFERENCES

As for project 2, during the three years of the project Dr Reeson and Professor Austin disseminated results of this research through numerous radio interviews, television appearances, assistance in the preparation of newspaper articles, through the running of annual National Wasp Workshops, the development of a website, and formal presentation of results at national and international conferences (also see under Project 2).

Conference Presentations

2002 “The status of *Vespula germanica* as an urban pest in Australasia” (A.D. Austin, M. Kasper & A. Reeson) *14th International Congress of IUSSI, Sapporo*.

2002 “Population ecology of an invasive wasp *Vespula germanica* in South Australia” (M. Kasper, A.D. Austin, & A. Reeson) *14th International Congress of IUSSI, Sapporo*.

2001 "Search for biological control of an introduced social wasp *Vespula germanica*" (A.F. Reeson and A.D. Austin) (August 2001) *Society for Invertebrate Pathology 34th Annual Meeting, Nordwijkerhout, the Netherlands.*

2001 "Microbial diversity associated with a social insect" (A.F. Reeson & A.D. Austin) *5th Invertebrate Biodiversity & Conservation Conference, Adelaide*.

2001 “What do social wasps feed on in South Australia? A comparison of *Polistes humilis* and *Vespula germanica* prey” (Poster: M. Kasper, K. Perry, A.F. Reeson & A.D. Austin) *5th Invertebrate Biodiversity & Conservation Conference, Adelaide.*

2000 "New initiatives toward the control of a social insect pest *Vespula germanica*" (G.M. Wood & A.F Reeson) *Australian Entomological Society Scientific Conference (June 2000), Darwin*.

2000 "Control of the European wasp *Vespula germanica* in urban South Australia" (Poster: A.F. Reeson, G.M. Wood, D.C. Hopkins & A.D. Austin) *Ecological Society of Australia Conference (November 2000) Melbourne.*

Publications

Reeson, A.F., Jankovic, T., Kasper, M.L., Rogers, S. & Austin, A.D. (submitted) Application of 16S rDNA-DGGE to examine the microbial ecology associated with a social wasp *Vespula germanica*. *Insect Molecular Biology*.

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APPENDIX - 1

Application of 16S rDNA-DGGE to examine the microbial ecology associated with a social wasp *Vespula germanica*

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Running title: Characterisation of an insect gut microflora

Abstract

Invertebrates host numerous bacteria, with interactions ranging from pathogenesis to symbiosis. While certain symbiotic relationships are well studied, little is known about the dynamics of these bacterial communities. Denaturing gradient gel electrophoresis (DGGE) was used to examine the bacterial microflora associated with a eusocial wasp *Vespula germanica*. DGGE profiles of larval guts revealed a variable microflora, suggesting that *V. germanica* is not dependent on a particular suite of mutualists. The variation in profiles was not related to season, nest size or macrohabitat. Sequences corresponding to *Lactococcus*, *Lactobacillus*, a novel *Leuconostoc* and two *Rickettsiella grylli* strains were obtained. DGGE proved a useful technique for characterising the wasp microflora. Given the importance of microbial communities to invertebrates there is much to be gained from the application of such techniques.

INTRODUCTION

Invertebrates provide a rich habitat for microorganisms. The interactions between invertebrate hosts and the microbes they harbour are numerous, and play a key role both in host physiology and whole ecosystem processes. Bacteria within the invertebrate gut are known to be important in the breakdown, mineralisation and cycling of a range of organic compounds (eg Cruden & Markovetz 1987; Kaufman *et al.* 1989; Breznak & Brune 1994) and are involved in methanogenesis and nitrogen fixation (Hackstein & Stumm 1994; Nardi *et al.* 2002). The gut microflora is also implicated in pheromone production (Byers & Wood 1981; Dillon *et al.* 2000), vitamin synthesis (Chararas *et al.* 1983), pesticide degradation (Boush & Matsumura 1967), and there is evidence that gut bacteria can prevent the growth of some insect pathogens (Dillon & Charnley 1995). As well as making use of bacteria in the gut lumen, it has been estimated that around 55000 insect species harbour endosymbiotic bacteria within specialised host cells; these bacteria provision their hosts with nutrients that are often lacking from their diets (Wilkinson 2001). Other intracellular bacteria include *Wolbachia*, which causes reproductive anomalies in a wide range of arthropods, and pathogens such as *Rickettsiella*. Manipulating endosymbionts has been proposed as a novel means of preventing insects from vectoring diseases (Beard *et al.* 2002).

Given the importance of these microbial associations to both invertebrates and the environment as a whole, it is remarkable how little is known about them. Relatively few studies have looked at whole microbial communities associated with arthropods. In one such study the collembolan *Folsomia candida* was found to harbour a specific microbial community from which non-indigenous microbes were swiftly removed (Thimm *et al.* 1998). The *Folsomia* gut flora is diverse, with 26 types of culturable bacteria isolated (Hoffmann *et al.* 1998). Honey bees *Apis mellifera* harbour a range of bacteria as well as yeasts and moulds in the gut (Gilliam 1997) In contrast the grasshopper *Melanoplus sanguinipes* contains an 'abundant but relatively simple' gut microflora consisting mostly of *Enterococcus* along with *Serratia*, *Pseudomonas* and *Enterobacter* (Mead *et al.* 1988), and there is evidence that related species of Orthoptera have similar microflora (Mead *et al.* 1988; Kaufman *et al.* 2000).

Most studies have examined the insect microflora by isolating and culturing its constituent organisms. As only a small portion of the bacteria associated with insects are likely to be culturable using existing techniques, alternative methods must be sought. Molecular techniques have been used to target the 16S gene, which codes for the small subunit of ribosomal RNA found in all prokaryotes; it is highly conserved but has distinct 'variable' regions which are useful in taxonomic studies. Group-specific rRNA targeted probes, G+C profiles and a 16S RFLP-like technique have been used to demonstrate that diet has an effect on the community composition of the cricket gut microflora (Santo Domingo *et al.* 1998a,b; Kaufman *et al.* 2000). Characterisation of 16S genes enabled Sandström *et al.* (2001) to identify three 'secondary' symbionts associated with the aphid-*Buchnera* symbiosis. Different lineages of the aphid *Acyrthosiphon pisum* showed great variability for the presence or absence of these bacteria, indicating the relationship is dynamic rather than constant (Sandström *et al.* 2001).

The objective of our study was to answer key questions about the ecology of an insect gut microflora, with the eventual aim of developing a novel approach for biological control. The eusocial wasp *Vespula germanica* (Hymenoptera: Vespidae) is an invasive pest in Australia, New Zealand, USA and South America. Previous attempts at biological control using pathogenic fungi have been unsuccessful, with hygienic behaviour within the nest preventing horizontal transmission of the pathogens (R. J. Harris pers comm). Larval wasps, which are reared in cells inside the nest, function as the gut of the colony, digesting food brought in by foraging workers and then regurgitating fluids which are subsequently passed around the colony (Spradbery 1973). The larval midgut is considerably enlarged as indigestible food remains are retained until pupation. *Vespula* wasps have a broad diet, including insects, carrion, fruit and nectar (Edwards 1980; Harris 1991) so they are likely to be exposed to a wide range of microorganisms.

We set out to test the hypothesis that there is a particular microflora associated with *V. germanica*, and to determine whether any such microflora varies with season and habitat. There are a number of practical implications. Firstly, if *V. germanica* depends on a particular microflora, then its disruption may provide a means of attacking the colony. Secondly, bacterial profiles may provide an easy means of distinguishing diseased nests, which would warrant closer examination. And there may be bacteria within the gut flora that themselves have potential as control agents. Denaturing gradient gel electrophoresis (DGGE) separates DNA fragments of the same length based on small differences in sequence. Applying this technique to 16S rDNA genes has great potential for profiling microbial communities, providing those communities are not overly complex, and has been widely used by microbial ecologists (Muyzer *et al.* 1993; Ovreas 2000). By using DGGE to examine the *V. germanica* microflora we aimed to both test our hypotheses about the nature of the microflora and determine the usefulness of the technique in insect-microflora systems.

RESULTS

DGGE profiles

DGGE of 16S rDNA fragments amplified by PCR showed a number of bands for each wasp nest sampled. There was considerable variation in profiles between nests. While some bands were widespread, occurring in approximately half of the profiles, most bands were restricted to one or a few nests. Replicate PCRs carried out on the same DNA sample appeared identical, indicating that the PCR reaction was reproducible (Fig. 1). Replicate samples taken from each nest showed some differences, although overall the profiles were very similar (Fig. 1). This indicates that not all wasp larvae harbour exactly the same bacterial genotypes. However intra-nest variation was far less than inter-nest variation, suggesting that the profiles represent real patterns.

DGGE profiles and ecological variables

A database containing DGGE profiles obtained from a total of 17 nests was created and used to compare banding patterns between different habitat types. For three randomly selected nests, two samples (taken from different groups of larvae) were included in the database in order to compare intra- and inter-nest variation. The database incorporated nests from a variety of habitats, collected throughout the summer and into autumn (Table 1). Similarity levels among each of the paired samples were far higher than among any other pair of samples, supporting the observation that inter-nest variation is much greater than intra-nest variation. Examination of the dendrogram produced from these data (Fig. 2) indicated that nests collected from the same habitat type or at similar times of the year did not cluster together.

Figure 1. Example of DGGE profiles of 16S bacterial genes contained in the guts of larval *Vespula germanica*. This gel tested the reproducibility of the DGGE profiles. Two DNA samples (labelled 1 and 2) were extracted from each of four wasp nests (K, D, B and H); replicate PCRs (labelled a and b) were then carried out on each DNA sample. The replicate PCRs were run in adjacent lanes, next to the PCRs from the other sample from each nest. Lanes 5 and 14 contain a reference sample (R). This gel was not used to construct the profile database.

Nest	Date	Traffic rate	Urban?	Altitude	No. of bands
A	29/01	51.1	U	sl	7
B	8/02	N	U	sl	7
C	15/02	30.5	R	hills	6
D	19/02	44.6	R	hills	8
E	27/02	24.7	U	sl	11
F	12/03	11.4	U	hills	8
G	12/03	55	U	sl	7
H	13/03	54	U	sl	11
I	16/03	N	U	hills	7
J	27/03	44	U	hills	13
K	27/03	81	U	hills	6
L	23/04	108	R	hills	6
M	7/05	38.8	U	sl	8
N	7/05	63.7	R	hills	5
O	15/05	27.2	U	sl	3
P	16/05	65.6	R	hills	6
Q	31/05	61	U	sl	9

Table 1. Locations of the nests used to compare DGGE profiles with macrohabitat variation. Date represents the date during 2000 on which each nest was destructively sampled; traffic rate represents the number of workers entering and exiting per minute, a measure of nest size (N – not recorded); urban? indicates whether the nests were located in a heavily built up area (U) or a rural area (R); altitude indicates whether the nest was found close to sea level (sl) or at >400m altitude (hills); number of bands distinguishable in the DGGE profiles when the samples were run simultaneously.

The number of bands present in a sample was not a function of sampling date ($F_{1,16}$=1.00; P=0.34). This indicates that the number of bacterial genotypes observed was not related to the age of the nest (nests are initiated in early spring, so date gives an estimate of the age of a nest). Whether a nest was found at high or low altitude ($F_{1,16}$=0.29; P=0.56) and in a rural or semi-rural area ($F_{1,16}$=2.30; P=0.15) had no effect on the overall number of bands. Traffic rate, which is an approximate measure of the number of individuals within a wasp nest, was also not related to the number of bands recorded from the DGGE profiles ($F_{1,14}$=0.59; P=0.46).

Band identification

The results of sequence alignments are shown in table 2. Sequences 2 and 3 were similar, both showing high levels of homology to *Rickettsiella grylli*. These sequences were obtained from different bands from nest E, and were also present in other nests. Duplicate sequences confirmed that the two genotypes were distinct. The most widespread band yielded a sequence corresponding to a strain of *Leuconostoc*; with only 90% homology to other *Leuconostoc*,

this is likely to represent a new taxon. The sequence obtained from band 1 showed a high level of homology to *Enterococcus*, but was chimeric with a short section of DNA corresponding to band 2.

DISCUSSION

These results suggest that there is no characteristic gut bacterial community associated with *Vespula germanica*. The nests showed a great variety of bacterial profiles, and the fact that there were differences between samples from the same nests suggests that, even within a nest, not all larvae harbour the same bacteria. The lack of a characteristic bacterial profile suggests that *V. germanica* is not dependent on a particular mutualistic microflora for its nutrition. A number of bacterial strains appeared widespread, but community composition varied between nests. In their review of the microbial ecology of the cockroach gut, Cruden and Markovetz (1987) suggest that insects can be loosely characterised into three groups according to their dependence on a microflora. They compare termites to ruminants, in that they are dependent on microbial processes in the gut, while insects which gain part of their nutrition from the gut microflora correspond to cecum-containing mammals. Cockroaches, which like *Vespula* have a broad diet, do not depend on their gut flora (Cruden and Markovetz 1987; Zurek & Keddie 1998), a situation that parallels the human digestive system.

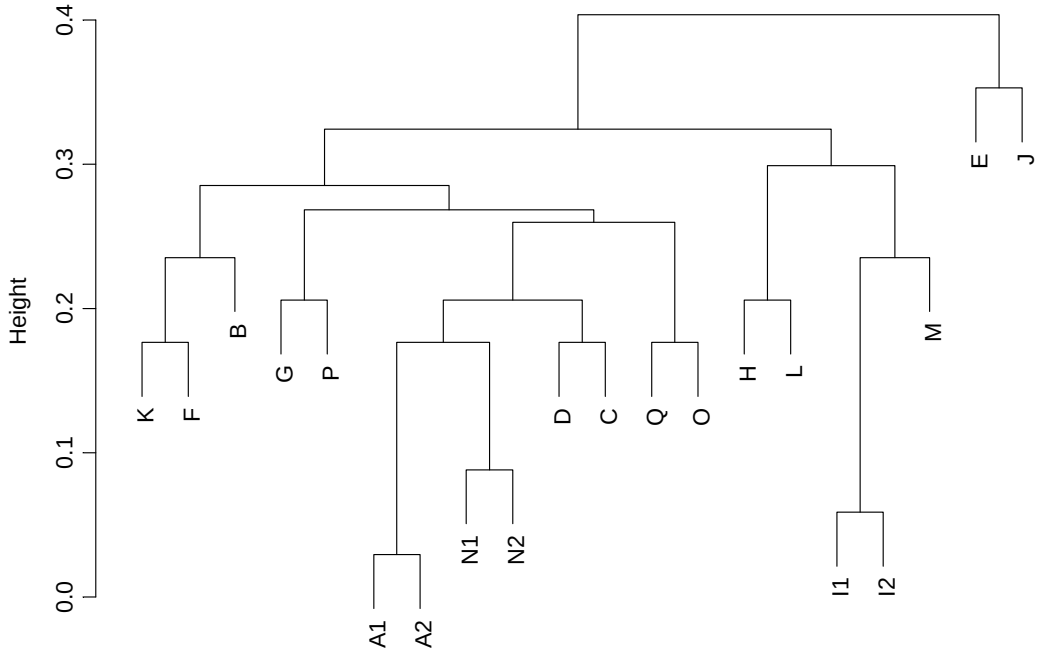

Figure 2. Clustering dendrogram of DGGE profiles from a sample of 17 *Vespula germanica* nests. Height represents dissimilarity. Labels relate to the nest site data described in table 1. For nests A, I and N profiles of both DNA samples taken from the nest were included, demonstrating that inter-nest variation is much greater than intra-nest variation.

Cluster analysis of the DGGE profiles did not reveal any relationship between bacterial communities and season or habitat. Nests collected at different times of the year did not cluster together, suggesting that wasp microfloras do not follow a particular ecological succession related to nest age or season. The lack of any relationship between the number of bands in a profile and the age of a nest suggests that the microflora has some degree of stability through time in any one nest. If the profiles simply represent bacteria brought in randomly from the environment, then older nests would be expected to contain greater diversity. Similarly, larger nests with more foragers would also be expected to harbour a greater range of bacteria, but again this is not supported by the data. The habitat differences recorded in this study (urban vs semi-rural and sea level vs hills) were somewhat crude but do reflect major ecological differences between the sites. However the small number of rural nests included in the analysis might have obscured any macrohabitat effect.

Band	FASTA nearest homology [accession number]	% homology (16S:27-534)	Sequenced from nests:-	Nests with corresponding bands
1	*Enterococcus* [EFA301831]	n/a	E	H, I, J, L, M, N
2	*Rickettsiella grylli* [U97547]	98	B, E	C, D, H, M
3	gamma proteobacteria related to *Rickettsiella grylli* [AF327558]	98	E, K, L	C, G, J
4	*Leuconostoc* [LMRRNAM]	90	K	A-N, P, Q
5	*Bacillus cereus* [BC16SR]	99	K	G, I, P,
6	*Lactococcus lactis* [AE006288]	99	H	J, L
7	*Lactobacillus kunkeei* [LSPRRNA]	99	K	E, F, H, I

Table 2. Results of sequence alignments against the EMBL database using the Fasta3 homology search program. The table indicates the nest samples each sequence was obtained from, and the nests which contained corresponding bands.

The variation in profiles may represent smaller scale variation in habitat, and with it the exploitation of different diets by the wasps sampled in this study. *Vespula* typically forage within a few hundred metres of the nest (Edwards 1980), and will return to sites where they have previously foraged successfully (Spradbery 1973). Therefore local habitat variation is likely to have a considerable effect on diet. The use of different food sources may promote the development of alternative bacterial communities. This appears to be the case in cockroaches and crickets, where microbial populations fluctuate in response to dietary changes (Kane & Breznak 1991; Kaufman *et al.* 2000). Diet is also known to influence the gut microflora in a number of aquatic invertebrates (Harris 1993). This apparent flexibility in bacterial communities may serve to buffer a host against changes in diet (Jones 1984; Kaufman *et al.* 2000). Whether these changes represent differential growth of intrinsic bacteria, or whether new bacteria are introduced with a change in diet, is uncertain (Kaufman *et al.* 2000). Extrinsic bacteria that are ingested by an invertebrate may be able to play a part in a stable community if they are routinely subject to differential survival and growth (Harris 1993). Diet might account for one exception to the overall lack of pattern in our data; nests C and D (Table 1), collected just 300 metres apart, yielded very similar bacterial profiles. This may be because workers from the two nests are foraging in the same area and bringing the same foods into the nest. However another possibility is that it could reflect genetic relatedness, as new queens typically disperse only a short distance from their parent nest (Crosland 1991). If microfloras can be passed between generations it could have many implications for wasp ecology.

DGGE appears a valuable tool for profiling the bacterial communities associated with arthropods, and is validated by the high levels of reproducibility seen in this study. The replicate PCRs carried out on the same DNA samples were identical, so DGGE is capable of producing consistent results from arthropod microflora. The different samples taken from the same nests showed some variation, although they were still broadly similar. This 'sampling error' emphasises the need to treat the data as an ecological sample rather than some sort of definitive molecular profile. In addition to bacteria, DGGE could also be extended to examine other organisms associated with insects such as viruses and protozoans by targeting other conserved genes.

The greatest advantage of the PCR/DGGE technique in the study of bacteria is that it potentially accesses the whole community, not just that portion that can be successfully cultured. Estimates of the proportion of bacteria that can be cultured using existing techniques vary from 0.001-15% (Amann *et al.* 1995). The ability to extract and sequence bands from gels provides a valuable tool, both for identifying key members of a community and screening for potential biocontrol agents. The usefulness of this technique is demonstrated by the identification of two *Rickettsiella* genotypes. Each genotype was sequenced from at least two nests, and corresponding bands were present in a number of other samples. However extrapolating to the presence of a genotype in other lanes needs to be done cautiously. It is possible for a band to contain more than one genotype, as very different gene sequences may happen to denature at the same point. And while these gels facilitate identification, *in situ* hybridisation studies will be required to confirm an active role for these bacteria in wasps. The wasp gut was also found to contain the lactic acid bacteria *Lactococcus*, *Lactobacillus* and *Enterococcus*, which ferment sugar to produce lactic acid, and in some cases other products including ethanol and acetate. They are frequently found in animal guts, where the nutrient-rich environment enables them to assimilate amino acids and vitamins that they do not synthesise

themselves. As aerotolerant anaerobes, lactic acid bacteria are able to live in most regions of the animal gut. The sequence showing 90% homology to *Leuconostoc* appeared widespread in wasps. The relatively low level of homology to previously described species suggests this is a new taxon, which may be an insect gut specialist. Similar sequences have been obtained from a related paper wasp *Polistes humilis* in South Australia (unpubl. obs.).

However while DGGE can yield much useful data, as with any other sampling technique it does have inherent biases. These biases can include differential lysis of cells during the DNA extraction and differential amplification of some genotypes in PCR. The competitive nature of PCR means that the absence of a band may not necessarily imply the absence of that genotype from a sample. The PCR reaction can also result in chimera and heteroduplex formation. Chimeras are formed when a partial length fragment from one organism binds to a full length fragment from another organism, resulting in a fragment of mixed sequence (Amann *et al.* 1995). On a DGGE gel chimeras form additional bands leading to an overestimate of diversity and the generation of erroneous sequences. The *Enterococcus* genotype sequenced in this study proved chimeric. Corresponding bands were present in a number of other samples, suggesting either that formation of this chimera is relatively commonplace, or that other genotypes were denaturing at around the same point. Heteroduplexes, formed when two different sequences of single stranded DNA anneal, will also form extra bands in a gel. Previous studies using molecular tools to identify bacteria from insects have relied on a random cloning and sequencing approach (eg Darby *et al.* 2001; Sandström *et al.* 2001). DGGE is much more efficient as individual genotypes can be picked from a gel, and different samples can be readily compared for the presence of the same genotype. Using this method it is much easier to detect rare genotypes. Band intensity can also convey useful information about the relative proportions of different genotypes in a community, provided the inequities of PCR are not forgotten.

'Although there are many scattered reports on the bacteria associated with insects, knowledge concerning the bacterial flora of insects in general is markedly scant.' That statement, made by Steinhaus in 1941, is still largely true today. While a great deal is known about certain bacteria associated with insects, community level knowledge remains limited. The microflora is involved in numerous processes impacting the host (including nutrition, pathogen resistance, toxin resistance, and communication) and its ecosystem (nutrient cycling, methane production). Since the microflora is not constant, there is the potential to mediate these impacts. Kaufman *et al.* (2000) state the importance of getting away from considering invertebrate microflora as some kind of ecological 'black box'. Rather it is a complex and dynamic community which is now amenable to close scrutiny. An improved knowledge of invertebrate microfloras will surely yield many dividends.

EXPERIMENTAL PROCEDURES

Wasp samples

Vespula germanica nests were sampled from a variety of sites in metropolitan Adelaide, South Australia, and the surrounding Adelaide Hills during summer 2000 (Table 1). The number of wasps in a nest was estimated by counting the number of workers entering and exiting over five one minute periods (after Malham *et al.* 1991). Nests were anaesthetised by pouring ether into the entrance, before being dug up and removed. Nests were placed into a freezer within an hour of extraction. Samples of 15 final instar larvae were collected randomly from throughout each nest. Larvae were dissected to remove the guts, which were pooled and homogenised. DNA was extracted from the homogenised gut samples using a commercial kit (Mo Bio ultraclean soil DNA extraction kit). Pooling the guts and treating them as for a soil extraction proved necessary as DNA yields were low and there was a large amount of organic matter in the wasp gut that inhibited subsequent polymerase chain reaction sequence amplification.

Polymerase chain reaction (PCR)

PCR was used to amplify a section of bacterial 16S rDNA present in the wasp guts and the surrounding tissues. All PCRs for DGGE were conducted using the following set of standard universal primers 27f (5'GAGAGTTTGATCCTG GCTCAG3') (Lane *et al.* 1985) and 519r (5'ATTACCGCGGCTGCTGG3') (Weisburg *et al.* 1991) targeting conserved regions within the 16S ribosomal gene. A GC clamp (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CCCGGGGG3') was added to the 5' end of the forward primer to stabilise the PCR product and prevent strand dissociation during DGGE. PCR amplification was performed in a total volume of 25µl containing 5µl DNA extract, 0.5µM of each primer, 180µM of each dNTP, 2mM $MgCl_2$, 1X reaction buffer and 1 unit of *Taq* DNA polymerase (SIGMA) using a thermal cycler (Eppendorf Mastercycler). Amplification was carried out under the following conditions, which were determined by a series of optimisation reactions: initial denaturation at 94°C for 4 minutes; 10 cycles of 94°C for 1 min, 65°C (with a touchdown step of -1°/cycle) for 1 min, 72°C for 1 min; 25 cycles at 94°C for 40 sec, 55°C for 1 min, 72°C 1 min; and a final elongation step at 72°C for 8 min.

Denaturing gradient gel electrophoresis (DGGE)
DGGE analysis of the PCR products was performed using a Bio-Rad Dcode system with 8% (w/v) acrylamide (acrylamide/bis-acrylamide 37.5:1[w/w]) gels containing a linear chemical gradient ranging from 40-55% (100% = 7M urea and 40% [v/v] deionised formamide). PCR products (20µl) were electrophoresed in 1x TAE buffer (40mM Tris, 20mM acetate, 1mM Na_2EDTA, pH 7.4) at a constant temperature of 60°C for 20 hours using a 60V current. A reference sample, consisting of PCR amplified *Bacillus weihenstephanensis* DNA isolated from a wasp nest, was run in at least two lanes on each gel to facilitate comparisons between gels. After electrophoresis the gels were stained for 10 minutes in SYBR gold (Molecular Probes) and photographed under a UV transluminator (Bio-Rad).

Reproducibility of profiles

To determine whether these DGGE samples were reproducible, two samples of 15 wasps were taken from each of four randomly selected nests and DNA was extracted from the larval guts. Two replicate PCRs were undertaken on each DNA sample. These replicate samples and PCRs were then run side by side on a DGGE gel.

Sequencing of fragments

Individual bands to be sequenced were picked by inserting a small pipette tip into the gel at each band of interest. The pipette tips were then placed into individual microcentrifuge tubes containing 50µl H_2O and held at 4°C overnight to elute the DNA. The pipette tip was then removed and PCR amplification was carried out on 5µl of the eluate. The 27f primer without the GC clamp was used for this amplification along with the 519r, with the same conditions as described above. Half of the resultant product was run on a DGGE gel to check that a single band had been amplified; if more than one band was observed, the process was repeated. The PCR products were then cloned into *E. coli* JM109 cells using the pGEM-T easy vector system (Promega). Plasmids were extracted using a purification kit (Qiagen) and sequenced using a Big-Dye sequencing kit (Applied Biosystems). Sequences were aligned with those in the EMBL database using a FASTA search algorithm (Pearson & Lipman 1988). All sequences were checked for the presence of chimeras using the CHECK_CHIMERA program from the Ribosomal Database Project (Maidak *et al.* 2001).

Band analysis

Samples obtained from 17 nests were randomly distributed over two gels, which were run simultaneously and in the same tank. Images of the gels were digitised using the Diversity Database software (version 2.1, Bio-Rad). Bands were identified by examining magnified images and absorption profiles, and the software was used to group bands from the same position in different lanes together. The use of two reference lanes, which yielded prominent bands at the top, middle and bottom of the lane (see figure 1), on each gel facilitated comparisons between the gels. Cluster analysis was used to look for groupings of profiles based on ecological variables (Krzanowski 1990). A dissimilarity matrix was calculated, based on the presence or absence of bands in each lane. For each pair of nests this represented the proportion of dissimilar bands out of the total number of bands. From this dissimilarity matrix a dendrogram was produced based on an agglomerative method of clustering. Agglomerative refers to the formation of a sequence of clusters using, in this case, the group average method, which plots the average of all dissimilarities between all pairs of nests. The dendrograms were examined for evidence of nests from similar habitats clustering together. In addition, the number of bands visible in each sample was calculated and linear regression analysis (GenStat) was used to test for effects of habitat, nest size and age. Two separate samples were run from each of three nests to compare intra- and inter-nest variation; the average number of bands was calculated for these paired samples.

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