The sympatric coexistence of two reproductively independent lines of the endoparasitic wasp *Venturia canescens*

by

Harry Leslie Scougall Roberts

Bachelor of Science (University of Adelaide)

Bachelor of Environmental Toxicology, Honours (University of South Australia)

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Department of Plant and Pest Science

Waite Campus

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"If we knew what it was we were doing,
it would not be called research, would it?"

Albert Einstein (1879-1955)

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Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photycopying.

Harry L. S. Roberts

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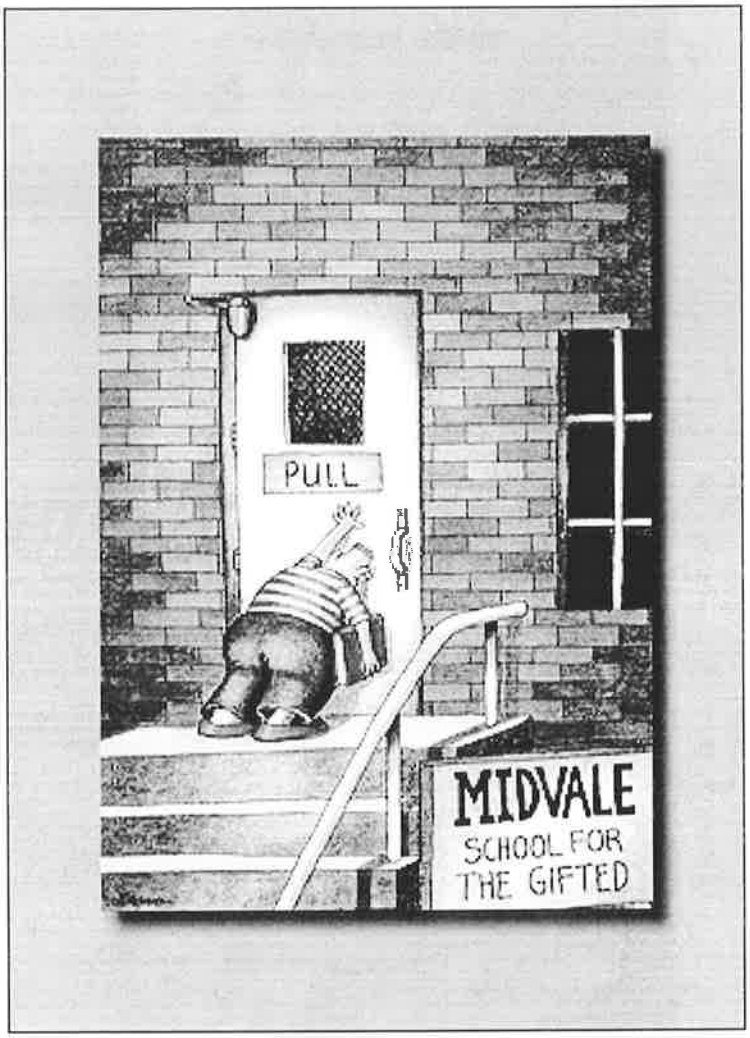
Chapter One

Overview of the Thesis.



Gary Larson and Steve Martin, (2003) *The Complete Far Side*. Andrews McMeel Publishing, New York

The principle of competitive exclusion (Volterra, 1926; Gause, 1934) states that it is impossible for two species that are limited by the same resource to coexist indefinitely. While mathematical models incorporating non-linear phenomena suggest that sympatric coexistence is possible under certain conditions (e.g. Levins, 1979; Armstrong and McGehee, 1980; Durret and Levin, 1998), the basic theory remains popular (Vandermeer *et al.*, 2002). In part, this is due to the numerous examples in the literature of competitive displacement of one species by another, but also because of the difficulty of demonstrating that there is no niche differentiation involved in those cases where species have been shown to be coexisting (see DeBach, 1966).

However, recent research has demonstrated that a laboratory culture of the asexual (=thelytokous) parasitoid wasp *Venturia canescens* contains two genetically distinct lines, coexisting on their host the flour moth *Ephestia kuehniella* (Hellers *et al.*, 1996; Beck *et al.*, 1999, 2000, 2001). The two lines are addressed as RP (repeat plus) and RM (repeat minus) for the presence or absence of a 54 base-pair tandem repeat sequence in the gene coding for a virus-like particle (VLP1) protein (Hellers *et al.*, 1996). The lines are genetically stable, and differ in a range of phenotypic characters, including ovarian morphology, calyx gland secretions and reproductive success (Beck *et al.*, 1999, 2000, 2001). Since the laboratory culture had been maintained without the addition of new stock for over 400 generations, the two lines must have been coexisting sympatrically while competing for the same resource.

The basis to the coexistence of the two lines appears to relate to differences in their reproductive success under single parasitism and superparasitism. In a simulation of laboratory conditions, Beck *et al.*, (1999) observed that when RM- and RP-wasps were allowed to compete for hosts for a 24-hour interval the RM-wasps produced significantly more offspring than the RP-wasps. However, under intra-line competition the RM-wasps produced significantly fewer offspring than the RP-wasps.

V. canescens lays eggs directly into the body of the larva of its host (=endoparasitic), where the developing parasitoid feeds on the haemolyph. When more than one egg is deposited into a host, it is described as superparasitised. However, irrespective of the number of eggs laid, at most a single wasp emerges from a host (= solitary parasitoid). When the time

interval between ovipositions is around three days or less, parasitoids fight for possession of the host using strong sickle-shaped mandibles to attack competitors, and the outcome is uncertain. For greater time intervals the older larva prevails, probably by suppressing the development of its younger adversary by anoxia (Fisher, 1961, 1963).

While the findings of Beck *et al.* (1999, 2000, 2001) suggest the system represents an exception to Gauss' Competitive Exclusion Principle, the following broad questions remain unanswered:

1) What is the basis of the higher reproductive success of the RM-strain under competing superparasitism?
2) How is the development of *V. canescens* in *E. kuehniella* modified under superparasitism compared to single egg parasitism?
3) Under what conditions will the reciprocal differences in reproductive success for the two strains lead to coexistence? Is the coexistence of the two strains an artefact of the laboratory rearing conditions, or can it occur in field populations?

The primary aim of the research undertaken during my candidature was to provide answers to these questions. A secondary aim of the research, through work performed in collaboration with a number of others, was to explore a fourth question:

4) What is the function of the VLP1 protein, and what role (if any) does it play in the phenotypic differences observed between the strains?

To achieve these aims, a number of specific issues within these broad questions were addressed.

I. The basis of the higher reproductive success of the RM-strain under competing superparasitism.

A possible explanation of the RM line's advantage may be based on the maternal phenotype and on both wasps laying multiple eggs in a single host, a common occurrence under laboratory culture conditions. Oviposition rates of up to 50 eggs per hour, continuing until the oviducts are largely

depleted of eggs, have been reported (Harvey *et al.*, 2001), including under conditions of intra-line superparasitism (Beck *et al.*, 2001). Regardless of the respective line, the eggs of the wasp ovipositing first hatch first. Interlarval physical combat between the siblings commences shortly after, so by the time the eggs of the second wasp hatch a number of the first wasp's offspring have already eliminated each other. The larvae of the second wasp at this point outnumber the larvae of the first wasp and so it is more likely that one of them will be the ultimate victor.

Compared to RP-females, RM-females delay ovipositing after being provided with access to hosts, and the RM-offspring display longer embryonic development times (Beck *et al.*, 1999), leading to a greater overall lag time between an RM-wasp being given access to hosts and her larvae hatching compared to an RP-wasp. Thus, the relative reproductive success of the two lines when competing for hosts may show an overall bias in the RM line's favour.

The general alternative explanation is that there is some physiological difference between the RM and RP lines that increases the probability of an RM larva winning a one on one encounter with an RP larva. Most plausibly, this would involve an advantage in one of the two previously identified modes of competition, either physical combat or physiological suppression.

Chapter 3 describes experiments conducted to distinguish between these two explanations. The study compared the reproductive success of the two lines when one egg from each line was laid into a single host, for a range of time intervals between ovipositions. The results showed that the RM-line won a significantly higher fraction (around 60%) of the overall contests, and further, that the competitive abilities of the two lines were not symmetric, indicating that the advantage of the RM-line relates to one-on-one interlarval competition rather than differences in maternal behaviour. Further, dissection of parasitoid larvae from superparasitised hosts indicated that most contests between competing larvae had occurred within the first 24 h of the eggs hatching, suggesting the advantage of the RM-line relates primarily to physical combat rather than to physiological suppression.

Chapter 4 details research to determine the nature of the phenotypic differences between the RM and RP larvae underlying the differences in

reproductive success described in chapter 3. The study examined the outcome of inter-larval physical combat under *in vitro* conditions. The results showed that the outcome depended on both the relative and absolute ages of the contestants, and that the competitive abilities of larvae from the two lines were not symmetric. In contests involving two larvae, at least one of which was newly hatched, the RP larva tended to lose, while if both larvae were at least 8-10 hours post-hatching when the contest occurred then the larger of the two larvae tended to lose. Thus, the higher reproductive success of the RM line under competing superparasitism with the RP line is due to a physiological difference between the newly hatched larvae of the two lines which results in an advantage to the RM larva, which occurs independent of the order or time interval between ovipositions.

II. The development of *V. canescens* in *E. kuehniella* under single egg parasitism and superparasitism.

One of the major aims of the research was to create a mathematical model of the interaction between the two strains. Having investigated the reproductive success of the two lines, it was next necessary to quantify the life history parameters of *V. canescens* in *E. kuehniella* under single egg parasitism and superparasitism.

Chapter 5 describes research documenting the influence of host mass and the time interval between ovipositions on the survival and development of larvae from both the first and second laid eggs in superparasitised *Ephestia kuehniella*, in this case for two competitively similar strains. As the time interval between ovipositions increased both overall and superparasitism success decreased, however time between, and order of, ovipositions had little effect on other developmental parameters. Adult size increased with host mass under both parasitism and superparasitism, while host mortality decreased with host mass under superparasitism. In addition, wasps emerging from superparasitised hosts were larger than wasps from parasitised hosts.

Chapter 6 describes research documenting the previously unreported phenomenon of egg dumping by an endoparasitoid wasp when deprived of hosts. These data also provide another of the sets of life history parameters

required to model the interaction between the strains, the lifetime fecundity of female *V. canescens*. Female *V. canescens* maintained without hosts began to deposit eggs onto the sides of the culture vessel on the day of eclosion. The maturation of additional eggs was not inhibited once the maximum oviduct egg load was reached but rather continued for the duration of the experiment (up to 39 days), at a rate of around 5.8% of the remaining unmatured eggs per day. When wasps were given access to hosts they matured additional eggs at an increased rate. Artificial damage to the ovipositor resulted in a reduced rate of egg maturation even though the oviducts were partly egg depleted, while damage to the auxiliary valvulae had no effect. These results suggest two conclusions. Under conditions of host deprivation the rate at which eggs are matured is determined by the rate of synthesis of precursors by the fat body that in turn is modified by feedback from the ovipositor, induced by physical stimulation. Further, the discarding of eggs is due to the involuntary unidirectional movement of eggs down the oviduct, facilitated by the ongoing maturation of additional eggs.

III. A mathematical model of the sympatric coexistence of two phenotypically distinct strains of *V. canescens*.

Having identified the basis of the differences in the reproductive success of the two lines under intra- and inter-line parasitism, and having quantified the relevant life history parameters, it was then possible to construct a mathematical model of the interaction between the two strains.

Chapter 7 presents the results of an iterative model that uses a range of experimental life history data to predict the stable composition of a mixed population of two lines displaying the laboratory phenotypes under different rates of superparasitism. Historically, the impossibility of showing that two species do not occupy separate niches has precluded any demonstration of sympatric coexistence in the field. The model predicts that sympatric coexistence of the two lines is possible when the overall rate of superparasitism is between 4 and 12% or greater. These values are within the rates reported for other solitary endoparasitoid wasp species in the field, and so demonstrate that the sympatric coexistence under natural conditions of two

species that display the phenotypes observed in the laboratory lines is, in principle, possible.

IV. The function of the VLP1 protein, and its role in the phenotypic differences observed between the strains.

The two *V. canescens* lines differ the presence or absence of a 54 base-pair tandem repeat sequence in the gene coding for a virus-like particle (VLP1) protein that is co-injected with the egg into the host (Hellers *et al.*, 1996). However, the role (if any) the VLP1 plays in the phenotypic differences observed between the strains is not clear.

Chapter 8 describes research on whether the *VLP1* gene is genetically associated with the phenotypes displayed by the RM and RP strains. The recent isolation of facultative sexual (arrhenotokous) and asexual *V. canescens* strains from the same location in Southern France enabled an investigation of the genetic basis for the observed phenotypic differences, by comparing the two asexual lines with the corresponding homozygous *VLP1* genotypes in arrhenotokous strains. This analysis showed similar patterns of morphological and functional differences exist in the ovaries of the two asexual *VLP1* lines and in the two homozygous *VLP1* genotypes from the field, suggesting that the *VLP1* gene alteration either causes the ovarian phenotype or is genetically closely linked to the putative gene. However, the *VLP1*-gene may not be the only gene contributing to the phenotypic effects observed in the asexual lines. Although the two VLP1-alleles segregate with the relative differences in the ovary distribution of eggs, the absolute egg numbers differ in the corresponding asexual and sexual genotypes. This suggests that an additional unlinked gene may be involved in the transfer of eggs from the ovarioles into the oviduct.

Chapter 9 details research that describes phenotypic changes in an RM strain following alteration in culturing conditions. Maintaining the RM line under conditions of low superparasitism resulted in rapid alterations in the phenotypic characters calyx eggload, egg maturation rate, and reproductive success under single egg and under competing superparasitism. The observed changes were not associated with changes in the RM-VLP1 allele, and in general are difficult to explain in terms of genetic change. The simplest

explanation for the observed phenotypic changes is that the RM phenotype is related to the action to a pathogen, specifically, an interaction between the RM genotype and a semi-permissive endosymbiont, whose transmission is vertical via maternal secretions from adult wasp to host and then horizontal from host to larval parasite. The data are also consistent with a mechanism based on maternal transmission of an inducible phenotype.

Chapter 10 discusses the findings of the research in the context of the two general explanations of the RM-phenotype, that the RM-phenotype is due to an endosymbiont or pathogen acting in some unknown fashion (Amat *et al.*, 2003), or due to pleiotropic effects of the allelic VLP1 gene locus (Beck *et al.*, 2000, 2001).

Other research conducted

During my candidature I was involved in several other projects, that were not directly related to the investigation of the sympatric coexistence of the two strains of *V. canescens,* but which led to a number of other publications. These are presented in *Appendix 1*, in order to give a full representation of the work I performed during my time as a PhD candidate.

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Chapter Two

Review of the Literature

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Endoparasitoid wasps are those that deposit their eggs inside the body of a second organism, typically an immature stage of another insect. The developing wasp larva selectively consumes its host, filtering nutrients from the host haemolymph in a fashion that is not immediately fatal. Since the host is of limited size and the wasp larvae are unable to move from one host to another, it is inevitable that only a limited number of parasitoids will be able to successfully complete their development inside a single host. Indeed, although numerous exceptions exist, in the majority of cases the sizes of the host and the parasitoid are such that a host will only support the development of a single parasitoid (Salt, 1961). However, no such limitation exists on the number of eggs that may be laid. When a host contains more than one egg, it is described as being superparasitised. This phenomenon has been frequently reported from field studies (eg Howard, 1897; Salt 1934). Of necessity, its occurrence often results in the deaths of some or all of the parasitoids.

For many years, and despite evidence to the contrary (Hill, 1926; Salt, 1932, 1934), it was widely believed that superparasitism was due to mistakes by the ovipositing female (van Lenteren *et al* 1978) and that any eggs so laid were wasted (Huffaker and Matsumoto, 1982). More recently, it has been recognised that superparasitism can be an adaptive reproductive strategy (van Alphen and Nell, 1982; Hubbard *et al*, 1987). In fact, it has been shown for some systems that the reproductive success rate under superparasitism is higher than under single parasitism (Baker, 1979).

A long-standing principle of evolutionary biology states that no two species can for long occupy the same environmental niche (Gause, 1934). However, recent research involving a laboratory colony of the endoparasitoid wasp *Venturia canescens* Grav. (Hymenoptera: Ichneumonidae) indicates that the population contains two reproductively independent strains, genotypically and phenotypically distinct yet coexisting sympatrically (Beck *et al*, 1999). Life history theory suggests that in a variable environment multiple evolutionary stable strategies can occur simultaneously in the same population (Stearns, 1992). The environment of the developing wasp larva is its host; this will vary qualitatively with the number and genotype of the larvae with which it has been parasitised.

This review focuses on aspects of intraspecific competition by the wasp *Ventura canescens* for its host *Ephestia kuhniella*. Following a description of the life cycle of the wasp under single-egg parasitism, the mechanisms of interlarval competition, and the factors influencing those mechanisms will be discussed. The final part of the review will examine the research that has been reported on competition within and between the two recently identified strains of *V. canescens*, genotypically and phenotypically distinct yet coexisting sympatrically.

LIFE CYCLE

Reproduction in the wasp *V. canescens* was until recently thought to be exclusively by thelytokous parthenogenesis, with haploid males both rare and sterile (Beling, 1932. Speicher, 1937). However, arrhenotokous individuals have also been discovered recently along the coast of southern France, typically in greater numbers than the coexisting thelytokous strains (Beukeboom *et al* 1999, Schneider *et al* in press).

In arrhenotokous strains, the meiosis and fertilisation follows the normal Hymenopteran pattern of oogenesis and syngamy. In thelytokous strains, the mode can be categorized as central fusion automictic parthenogenesis (Beukeboom and Pijnacker, in press), with two haploid pronuclei that segregated at meiosis I fusing following meiosis II to restore diploidy.

Since crossing over can occur prior to meiosis I this mode of reproduction has two important consequences. Over successive generations the degree of heterozygosity in a lineage will decrease the more distal the loci are to the centromere. In addition, a group of siblings may be genetically different from both their mother and each other. Thus, evolutionary adaptation in thelytokous *V. canescens* lineages should be possible from existing genetic diversity, albeit in a limited and possible irreversible form (Slobodchikoff 1983).

The reproductive tract of the female wasp extends almost the entire length of the abdomen. The paired ovaries each consist of proximal ovarioles and a long basal oviduct, connected by a calyx gland. Each ovariole contains a sequence of progressively developing germ cells. At the proximal end is a

group of resting oogonia, followed by dividing oogonia. These are followed by a long series of primary oocytes in a progressive sequence of prophases, alternated with groups of large nurse cells and surrounded by follicle cells. By the time each oocyte reaches the base of the ovariole it has absorbed all the cytoplasm from the nurse cells, has acquired a chorion laid down by the follicle cells and is either in or approaching the first metaphase. The eggs, which are still mature oocytes until physically activated by oviposition (see below), then pass through the calyx gland where they are coated and surrounded by calyx fluid before passing into the oviduct for storage. Oviduct eggs are all in early first anaphase, lack cellular accessories, and have the shape of a long and slightly curved cylinder (Speicher, 1937).

Prior to an oviposition attempt by the adult wasp an egg is moved from the oviduct to the top of the ovipositor by a lateral flexing motion. The ovipositor penetrates the cuticle of the host caterpillar and the egg, along with a range of maternally derived secretions, is injected directly into the host's haemocoel (Rogers, 1972). The secretions are involved in protecting the parasitoid from the host's immune response (e.g. Feddersen, *et al* 1986; Beck *et al* 2000) and in marking parasitised hosts (e.g. Harrison *et al* 1985; Marris *et al* 1996). However, in contrast to a number of other Hymenopteran parasitoids (e.g. Dover *et al* 1987, Strand & Dover 1991) a role in modifying host development appears unlikely (Harvey 1996).

Immediately after oviposition the parasitoid egg starts to absorb fluids from the host's haemolymph and swells, increasing in volume by a factor of five by the time the eggs hatch (Corbet & Rotheram 1965). Simultaneously the first meiotic division is resumed (Speicher 1937), possibly triggered by changes in osmolarity, although a protease activity may also be involved, and embryogenesis fails soon after, unless mechanical deformation of the egg has also occurred (Sander & Feddersen 1985). Following the second meiotic division two diploid nuclei are formed. After several divisions the products of the polar nucleus degenerate and the cleavage nuclei eventually form the embryo (Speicher 1937). At a temperature of 25 °C the time for embryonic

development to be complete can range from 60 to 90 hours with a median value of around 68 hours.

Following hatching the first instar larvae live freely within the haemolymph of the host (Corbet & Rotheram 1965). Upon hatching the first instar wasp larvae do not immediately kill the host but rather selectively feed in a fashion that allows the host itself to continue to feed and grow. If larvae hatch in a host younger than the final instar, the parasitoid will feed and grow very slowly, remaining in the first instar until the host development reaches the mid final instar. At that point feeding and growth rates increase rapidly, and the larvae reaches the final (fifth) instar around 6 days later, at which stage most of the host tissues have been consumed (Corbet & Rotheram 1965, Harvey 1996).

What determines the switch from slow to rapid development is unclear. Corbet (1968) measured changes in freezing point depression, and in the concentration of proteins and amino acids in the haemolymph throughout larval development, and found that the onset of rapid parasitoid growth coincided with the point at which there was a substantial increase in protein concentration and decrease in both freezing point depression and amino acid concentration. In a second series of experiments, larval feeding rates were measured *in vitro* under different solute concentrations within the range of *E. kuehniella* haemolymph. It was found that, in general, feeding rates were lower the higher the solute concentration, suggesting that the critical stimulus for the initiation of rapid growth was the fall in solute, most notably amino acid concentration.

However, research into the rearing of *V. canescens* larvae *in vitro* in synthetic media suggests rising lipophorin concentration may be involved in the initiation of rapid growth. It was found that larvae did not progress beyond the first instar unless lipophorin was a component of the media, and that development time decreased as lipophorin levels increased up to a concentration of three mg/ml (Nakahara *et al* 1999, 2000). While typical lipophorin concentrations in *E. kuehniella* are not known, in the lepidopteran species *Manduca sexta* and *Bombyx mori* lipophorin occurs in the range one-five mg/ml (Tsuchida *et al* 1998; Beckage & Kanost 1993), suggesting that

three mg/ml is similar to the normal *in vivo* concentration. Further, in the four days following the fourth/fifth instar ecdysis, lipophorin levels rose from around one mg/ml to 3.5 mg/ml in *M. sexta* (Beckage & Kanost 1993). However, similar changes were also reported for *M. sexta* following the third/fourth instar ecdysis (Tsuchida *et al* 1987). If the same pattern were also true for *E. kuehniella* this would imply that lipophorin concentration might be a necessary but not sufficient precondition for the initiation of rapid growth in *V. canescens*.

A second group of candidates that drive development are the host-derived hormones, in particular the ecdysteroids. Under *in vitro* conditions using synthetic media Nakahara *et al* (1999, 2000) found the fraction of larvae reaching the second instar increased from less than 5% to almost 95% by the addition of one µg/ml 20-hydroxy-ecdysterone (20-HE), while the time required decreased from 31 to 11.7 days. When the concentration was increased to 10 µg/ml, the fraction of second instars dropped to 40 %, with a high incidence of malformed larvae, implying the 20-HE is acting as a moulting hormone rather than a nutritionally beneficial substrate, as has been suggested for the parasitoid *Campolitis sonorensis* by Ho & Vinson (1997). Rather, the data indicate that *V. canescens* utilize exogenous ecdysteroids, either directly to stimulate development or indirectly to stimulate endogenous ecdysteroid synthesis. Juvenile hormone was found to enhance larval growth rates, but had little or no effect on the fraction of larvae reaching the second instar (Nakahara *et al* 1999, 2000).

After reaching the fifth instar the wasp larvae pupate, typically around 14 days after oviposition. An occasional parasitoid will leave the remains of the host but the majority remains inside the host cuticle. The larva spins a cocoon, discharges a dark meconium and moults into a pupa. After another seven days the pupa moults into an imago and finally, around 25 days following oviposition the adult wasp emerges (Corbet and Rotheram 1965, Salt 1977).

V. canescens are ready to oviposit immediately after emergence and continue to mature eggs for a considerable portion of their lifetime [= synovigenic (Flanders1950)] (Kreiger 1927, Diamond 1929). The eggs are

small and yolk deficient [= hydropic], produced without the female requiring an extrinsic protein source. Therefore adults feed exclusively in order to maintain metabolic function, on sugar-rich foods such as nectar (Harvey *et al* 2001). At eclosion a female has around 50-60 mature eggs in her oviducts (Fletcher *et al* 1994) and for at least the first few days continues to produce mature eggs at a rate of 40-50 per day (Trudeau & Gordon 1989). Egg load varies with body size (Ahmed 1936). In trials where wasps were provided with excess hosts, some individuals produced over 400 offspring during their lifetime (Harvey *et al* 2001).

Frequent access to a sugar source is critical to adult survival. In trials conducted at 25 °C wasps starved from eclosion lived on average three days, while wasps that were alternately provided with honey for 24 h then starved for 48 h lived on average only five days. Further, lifespan was little affected by whether the wasps had access to hosts or not (Harvey *et al* 2001).

INTERLARVAL COMPETITION

Since ovipositing *V. canescens* females do not engage in ovicide of earlier laid eggs (Salt 1961, Fisher 1961), if two or more eggs are deposited into a single host the two parasitoids must compete with each other for possession of the host. Two modes of competition have been identified, with the dominant mode depending on the age difference between the two parasitoids. There is no evidence for any interactions between parasitoid eggs before they hatch.

The first mode of competition involves physical combat and can occur if the age difference between the larvae is three days or less (Fisher 1961). First instar larvae possess well-developed mouthparts, and upon meeting one larva bites through the cuticle of the other, allowing haemolymph to bleed from the wound. If not killed outright, the wounded competitor then ceases to move or feed and is subsequently overcome by the host's immune response (Fisher 1961, Salt 1961, Mackauer 1990). In addition, under *in vitro* conditions Marris & Casperd (1996) found that the greater the age difference between the larvae, the greater was the advantage of the younger competitor in terms of proportion of fights initiated, and number and duration of bites inflicted.

The second mode of competition can occur if the age difference between the larvae is around three days or greater, and involves physiological suppression of the younger larva by the older (Simmonds 1943). In this situation the younger larva either fails to hatch or, upon hatching fails to feed or grow (Salt 1961). A number of mechanisms have been suggested by which an older parasitoid larva might suppress a younger competitor, including starvation, toxic secretions and haemolymph changes (reviewed in Vinson & Hegazi 1998). In the case of *V. canescens,* the cause is most likely asphyxiation (Fisher 1961, 1963).

In one series of experiments, Fisher (1961) ligated parasitised hosts with cotton thread such that one parasitoid was trapped in each half of the host without preventing the free circulation of the host's haemolymph. The pairs of parasitoids differed in age by three, four, five or six days. The hosts were reared in 50 % oxygen for seven days after the eclosion of the younger parasitoid and then dissected. On no occasion was the younger larvae suppressed. This implies that physiological suppression is not due to a secretion by the older larvae, nor by the control of critical nutrients.

In a second series, hosts were superparasitised with a time difference of between three and six days, reared variously under an oxygen content of 5 to 50 % for seven days, after which the larvae were dissected out (Fisher 1961). Under low oxygen conditions all larvae were suppressed. Under atmospheric oxygen conditions the older larvae won by physiologically suppressing its opponent if the time difference was four days or more. When the time difference was three days the older larva won most contests by suppression, but lost a minority through physical combat. However, under high oxygen conditions the younger larva was never suppressed but usually won the contest by physically attacking the older. This strongly suggests that the basis to the physiological suppression of a younger larva by an older is anoxia and that it occurs when the age difference is three days or more.

In a third series of experiments Fisher (1963) examined the change in the rate of oxygen consumption of larval *V. canescens* with age under *in vitro* conditions. Newly hatched larvae consumed oxygen at a rate of around 0.03 ml/hour, with the rate slowly increasing to 0.06 ml/hour after 2 ½ days. The

rate of oxygen consumption then increased rapidly to around 0.15 ml/hour for three-day-old larvae and 0.35 ml/hour for four-day-old larvae. Thus, the age at which oxygen consumption increased coincides with the point at which older larvae are able to suppress their younger competitors.

In a fourth series of experiments, Fisher (1963) investigated the *in vivo* survivorship of *V. canescens* eggs and larvae under reduced oxygen conditions. Mortality was found to increase as oxygen partial pressure decreased, and mortality was greater among eggs and newly hatched larvae than late first instars. In a final series of experiments, the differential survival of eggs and larvae under extremely low oxygen tension was examined by sealing them *in vitro* under liquid paraffin. All eggs and newly hatched first instar larvae died within 24 hours, while late first instars and older larvae remained alive, moving and feeding on the liquid paraffin for over four days. This implies that eggs and newly hatched larvae have a higher absolute requirement for oxygen than later immature stages.

Together these data provide strong evidence that in the case of *V. canescens* the physiological suppression of a younger larva by an older larva is through asphyxiation. The effect is a consequence of the egg or young larva having a relatively higher requirement for oxygen, while the older larvae is both more tolerant of a low oxygen concentration and also able to consume the greater fraction of the available supply due to its larger size. This effect can occur when the age difference is around three days and greater (Fisher 1961, 1963).

Based on what is known of the two modes of competition, physical combat and physiological suppression, it is possible to predict the relative survivorship of the older and younger larvae in a host superparasitised at different time intervals. When the age difference is close to zero, the contest is decided by fighting, the larvae are of similar size and so the relative success is equal. As the age difference increases to around 60 hours the contest is still determined by fighting, but the younger larva has a progressively increasing advantage and so the relative success rate of the younger compared to the older larva should progressively increase. At an age difference of greater than around 60 hours physiological suppression should start to influence the

outcome, with the fastest developing older larva able to suppress the slowest developing younger larva, and the relative success rate of the younger larva should start to fall. By an age difference of around 84 hours the slowest developing older larva should be able to suppress the fastest developing younger larva, and the relative success rate of the younger larva should fall to zero. The general shape of the expected curve is shown in Figure 1.

The relative survivorship of older and younger larvae under superparasitism, with different age differences and each wasp laying a single egg, was investigated experimentally by Sirot (1996). The results are shown in Figure 2. In the study a difference between two strains in the colouration of the adult abdomen was used to identify the emerging wasps. Interestingly, although no genetic analysis was performed, the same differences in abdominal colouration are observed in the coexisting strains to be discussed later.

While prediction and experiment are in broad agreement, a notable difference is the absence of any advantage to the younger larva over the first 48 hours, indicating that the model is incomplete. There are a number of possible explanations.

The susceptibility of a larva to a wound may decrease with age, so that a bite of sufficient severity to kill a newly hatched larva may not be severe enough to kill an older larva. Marris & Casperd (1996) measured the order, number and duration of bites, and made no estimate of their severity. While Fisher (1961) concluded from the dissection of hundreds of superparasitised hosts that if a larva was bitten it would invariably die, it must be noted that much of his data were derived from parasitism of a fresh host by mechanical injection of eggs dissected out of a naturally parasitised host. As such, the host in which the larvae developed would not have been exposed to any immune-suppression by maternal secretions, and so the larvae may have been exposed to a far more hostile environment than would naturally occur. For similar reasons physical combat may not determine the outcomes in all cases, even though both larvae are active. While Fisher (1961) reported that when the age difference between larvae was less than 48 hours physical combat always occurred, in most cases with 24 hours of the younger larva

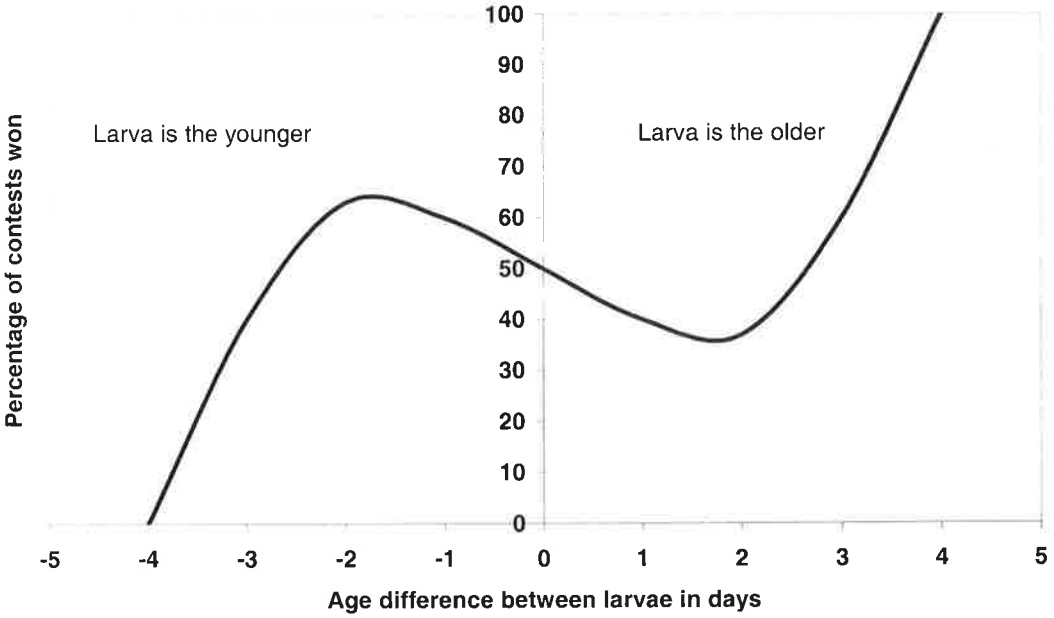

Figure 1. Predicted percentage of interlarval contests won *in vivo* by *V. canescens* larvae as a function of the age differences between the two competing larvae.

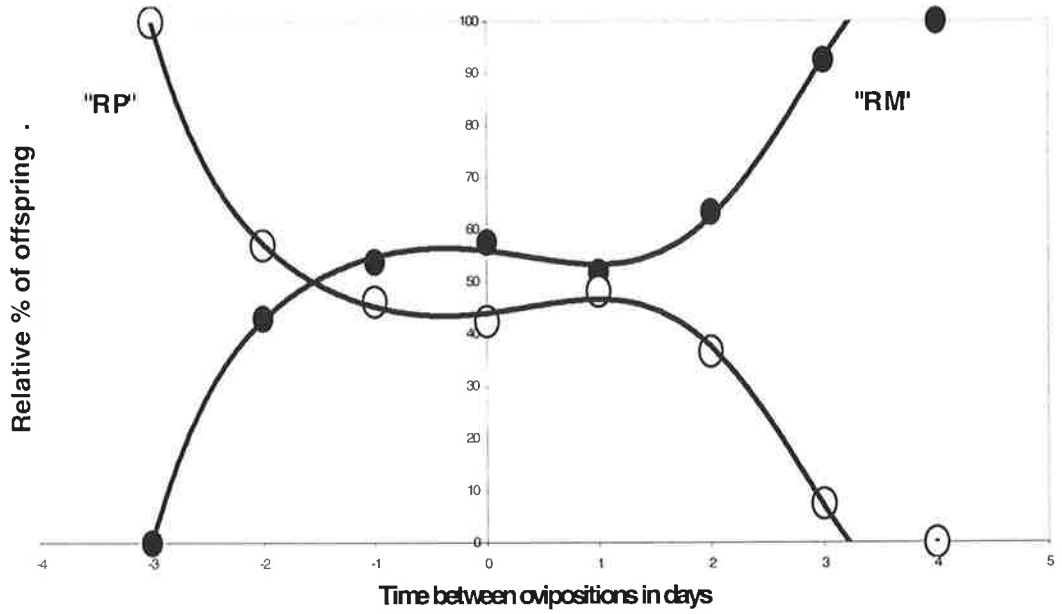

Figure 2. Relative percentage of emergent *V. canescens* offspring as a function of the time between oviposition events. Negative times indicate that the "RM" wasp was introduced second. Closed circles signify "RM" offspring and open circles signify "RP" offspring (Sirot, 1996)

hatching, the possibility that it is not always decisive cannot be discounted. If so the advantage should lie with the older larva which, being developmentally more advanced, would begin the rapid feeding phase earlier and ultimately starve its younger competitor.

Another possibility is an interaction between suppression by anoxia and physical combat. If the lack of oxygen experienced by a larva three days younger than its competitor is sufficient to completely prevent movement or feeding, it might be expected that for a smaller age difference the degree of oxygen lack would be sufficient to inhibit activity and thus reduce a larva's ability to attack or evade attack. However, the range of larval age differences over which such an effect would occur is likely to be narrow. Physiological suppression is argued to depend on two effects; the rapid increase in oxygen consumption by the older larvae at the time of the first/second instar moult coinciding with a high absolute requirement for oxygen by an embryo or very young larva. However there is little change in either consumption of, or absolute requirement for oxygen between the ages of 6-48 hours and so, within that range, it is difficult to see how any inhibitory effect from reduced oxygen levels would offer a significant advantage to the older larva.

A final possibility is that the outcome of interlarval combat depends on both the relative and absolute ages of the larvae involved, with a newly hatched larva being more vulnerable to attack than a larva six hours post hatching. There are several possible mechanisms for such an effect.

The chance of a larva winning a fight would seem most likely to depend on three factors: the physical qualities of its mandibles that enable it to puncture an opponent's cuticle, the physical qualities of its own cuticle that allow it to avoid being punctured in turn, and the relative speed and agility of the larva, allowing it to attack or avoid attack. In many insects, the mandibles and cuticle continue to harden for some hours after hatching. Whether this is true of *V. canescens* is not known, but if it is so it would likely result in a newly hatched larvae having a relatively lower offensive or defensive capability.

It is also possible that if combat occurs before the young larva has had an opportunity to feed, then it lacks nutritional reserves, quickly tires and becomes vulnerable.

Finally, a newly hatched larva may be vulnerable to locally induced anoxia. When fights are observed in vitro, the two larvae frequently actively manoeuvre for a position in very close proximity for tens of minutes before the first bite is inflicted (Marris & Casperd 1996). Thus it is possible that the oxygen content of the haemolymph surrounding the two larvae is substantially reduced, and that the newly hatched larvae, due to its higher absolute requirement for oxygen, is sufficiently inhibited to render it vulnerable.

In the Marris & Casperd (1996) study, the age of the younger larva was 72 hours post oviposition. Given that embryonic development time can be as short as 60 hours (Corbet & Rotheram 1965) the larvae may have been any age between zero to 12 hours post hatching. If the outcome of physical combat is in part dependent on the absolute age of the younger larva, then the results of the Marris & Casperd (1996) study would be a product of the age distribution of the individual larvae used, and the outcome of larval competition *in vivo* a function of the probability distribution over time of two larvae meeting.

TWO COEXISTING STRAINS OF *V. CANESCENS*

Hymenopteran endoparasitoids produce a range of secretions which are injected into the host during oviposition (Beckage 1997) that act to facilitate the development of the young wasp by interfering with the host's defense system and also other host functions (Quicke 1997).

In the case of the endoparasitoid wasp *V. canescens* these secretions include virus-like particles (VLPs) which are produced in the calyx tissues of the reproductive tract. As the mature oocyte passes through the calyx on its way from the ovariole to the oviduct, it is coated in very large numbers of VLP proteins which bind to the chorion (Rotheram 1973). It was found that five major VLP proteins exist, ranging in size from 35 to 80 kDa (Feddersen *et al* 1986). The DNA of one of the five (coding for the 40 kDa protein, named the VLP1) was cloned and characterised, and it was found that it occurred within the laboratory wasp population in two allelic variants, differing by the presence or absence of an 54 bp tandem repeated sequence. Interestingly, all wasps analysed were found to be homozygous for one of the alleles (Hellers *et al* 1996). Because of this, in combination with the population's asexual mode of

reproduction, it was possible to establish two separate clonal lines ("repeat plus", RP and "repeat minus", RM) based on the allelic variation.

Following this the two wasp strains were tested for stability in the VLP1 gene over successive generations. No alterations were detected in either gene. The genomic DNA was then examined using RAPD-PCR. From the 23 random primers tested, 90.5 % polymorphic and 9.5 % monomorphic scorable bands were obtained for the two strains. Together, these results indicate that the two strains are stable and have been separated for enough time to accumulate other DNA polymorphisms, and that the presence of the two VLP1 alleles in the laboratory population was not due simply to mutation pressure (Beck *et al* 1999).

A number of phenotypic differences between the two strains were also observed. Initial attempts to culture the RM strain failed several times, with development of the individuals frequently failing at the pharate stage (M. Beck, *pers. com.*). Once established, RM wasps continued to produce fewer offspring than RP wasps under conditions of intrastrain superparasitism. However, under interstrain superparasitism, RM wasps produced more offspring than RP wasps (Beck *et al* 1999).

Examination of the reproductive tracts of individuals from the two strains revealed further differences. The calyx glands of RM wasps were larger than those of RP wasps, with extended VLP-filled membrane systems, and the amount of secreted VLP1 protein in the calyx was reduced for the RM wasps compared to the RP wasps. The transfer of eggs through the calyx to the oviduct also appeared to have been affected by these differences. When 7 day old wasps were dissected, RM wasps were found to have significantly more eggs in the calyx and significantly fewer in the oviduct, compared to RP wasps. Finally, it was observed that RM eggs were both larger, and took longer to hatch, than RP eggs (Beck *et al* 1999).

Since the two wasp strains are genetically stable, and the original laboratory culture had been maintained without the addition of new stock for many years, the two strains had been coexisting sympatrically. Given the pronounced differences in morphology and reproductive success between the two strains this further suggests they coexisted as a balanced polymorphism.

One issue raised by this research is the genetic basis of the RM phenotype, specifically, whether the phenotype is genetically linked to the VLP1 gene and if so, whether the phenotype is a result of the pleiotropic effects of the VLP1 gene alone, or an allelic combination of the VLP1 gene and (at least) one other.

The isolation of sexually reproducing strains of *V. canescens* from the same location in southern France, which contain both alleles of the VLP1 gene (Malmberg *et al* 2000) provides an opportunity for this issue to be investigated. Lines homozygous for the two VLP1 alleles can be extracted from an interbreeding mixed sexual colony, and then the correspondence between the VLP1 allele and the relevant morphological and reproductive phenotypes determined. Since the RM and RP homozygous sexual lines will have been derived from a single population containing females heterozygous for the VLP1 gene, it is reasonable to assume that any genes not linked to the VLP1 gene locus will occur at similar frequencies in offspring from the separated lines. Thus, any correspondence observed between VLP1 allele and phenotype would constitute evidence that the VLP1 gene alteration either causes the phenotype or is genetically closely linked to the putative gene.

The second issue raised is the function of the VLP1 protein and the relationship between the two allelic forms and the corresponding phenotypes. Potential similarities between the deduced VLP1 amino acid sequence and other proteins were investigated by screening existing DNA and protein sequence computer libraries. Significant similarities were found between the VLP1 and a vertebrate protein belonging to the family of phospholipid hydroperoxide glutathione peroxidases (PHGPX). However, several key amino acids typically present in the catalytic center of vertebrate peroxidases (Brigelius-Flohe *et al* 1994) are not present in the VLP1. Further, *in vitro* reactions using VLP1 proteins found no significant peroxidase activity. Thus, an enzymatic role for the VLP1 in reducing hydroperoxides of phospholipids appears unlikely (Hellers *et al* 1996). An alternative is that the VLP1 protein is involved in the non-enzymatic binding and masking of modified lipid moieties in the VLP-membrane. PHGPX reduces peroxides at the expense of GSH

(equation 1) which is regenerated in a reaction catalysed by GSSG reductase (equation 2) (Thomas *et al* 1990).

$ROOH + 2GSH \longrightarrow ROH + GSSG + H_2O$ (equation 1)

$NAPDH + H^+ + GSSG \longrightarrow NADP^+ + 2GSH$ (equation 2)

However, the VLP1 protein occurs extracellularly, in the lumen of the calyx of the wasp and in the haemolymph of the host following oviposition, so that the substrates such as glutathione required for continued enzymatic activity are likely to be absent, making an enzymatic function inappropriate. In addition, the VLP1 protein is secreted in very large quantities (Rotheram 1973). Thus the VLP1 protein may act to reduce oxidized lipids, but with an individual protein able to act only once.

Under this model, the physiological function of the VLP1 protein is to mask oxidised lipids in the membranes of the calyx and on the surface of the egg. The RM allelic form may be less able to fulfil this role, resulting in the observed calyx morphology as well as other unidentified molecular consequences on the egg or larvae following oviposition. Until the physiological basis to the reproductive differences between the two genotypes is understood, any molecular explanation of the differences would be entirely speculative. However, one general scenario could be that the damage that occurs to the calyx of the RM genotype causes a significant reduction in the concentration of other calyx gland secretions in the lumen that in turn have consequences during embryogenesis or larval development.

The third question raised by the research is the basis for the differences in reproductive success between the two strains under intra- and inter- strain superparasitism.

In the original Beck *et al* (1999) study, when an RM and an RP wasp were allowed simultaneous access to hosts for 24 hours, there were almost twice as many RM as RP offspring. Noting that the embryonic development period for the RM strain was longer than for the RP strain, it was suggested that the effect was due to the RM larvae hatching second and so accruing the advantage of being the younger larvae under physical combat (as per Marris & Casperd 1996). Ignoring for the moment the difficulties in reconciling the *in vivo* data of Sirot (1996) and the *in vitro* data of Marris & Casperd (1996), a

weakness of this explanation is the magnitude of the apparent RM strain advantage of two to one, since for a 24 hour age difference Marris & Casperd (1996) reported a much smaller younger larval advantage of four to three.

Since the differences in the duration of embryonic development alone appeared to be too small to account for the magnitude of the RM strains' competitive advantage, it was hypothesised that the effect was enhanced by differences in oviposition behaviour between the two strains. It has been shown that the rate of searching behaviour (Trudeau & Gordon 1989), oviposition (Sirot *et al* 1997), and the likelihood of winning an agonistic encounter (Hughes *et al* 1994) all increase with egg load. RM wasps, with fewer eggs in their oviducts (Beck *et al* 1999) may thus tend to delay oviposition compared to RP wasps. This was investigated by allowing wasps of both strains simultaneous access to a patch of hosts for three hours with each stinging attempt combined with cocking behaviour (as per Rogers 1972) recorded as an oviposition event. It was found that over the first two hours the RP wasps made significantly more oviposition attempts than RM wasps, but after two hours the situation was reversed, with the RP wasps making fewer, although the cumulative number of attempts by RP wasps remained higher. An important point to note is that for both strains, the number of oviposition attempts was many times greater than the number of hosts. Clearly, both strains are prepared to lay multiple eggs into a single host.

In a second experiment the relative reproductive success of the two strains under competing superparasitism was investigated, with wasps allowed access to hosts for one or three hours. The results mirrored those for oviposition rates, with RP wasps producing more offspring under the one-hour access condition while RM wasps produced more offspring under the three-hour access condition (Beck *et al* 2001). These experiments demonstrated additional functional differences between the strains, and suggest that the timing of egg deposition is a relevant factor in the relative success of the two strains. However, even the combined effects of delayed oviposition and a longer embryonic developmental time would not seem to be able to increase the age difference between RP and RM larvae enough to explain the

observed two to one RM advantage purely on the basis of a younger larval advantage in physical combat.

Since the data suggested that the timing of egg deposition is a relevant factor in the relative success of the two strains, the effects of differences in the timing of oviposition were subsequently further explored.

Beck *et al* (2000) examined the effect on relative reproductive success of allowing one of the two strains a head start. Here, one wasp was given access to 25 hosts for seven hours before the second wasp was added, after which both wasps were allowed to search for and parasitise hosts a further 24 hours. When the RM wasp was given the head start there was no significant difference in the number of offspring from each strain, while when the RP wasp was introduced first, the RP strain produced around twice as many offspring as the RM strain. If it is assumed that the combined effects of delayed oviposition and a longer embryonic development time equate to around a seven hour lag then the first result is consistent with the young larval advantage explanation, however, the latter result is contrary to it, and rather difficult to explain, unless it is assumed that the advantage of a younger larva only exists across a range of age differences of a few hours. However, it should be noted that the number of replicates was low (two) and the data may merely reflect within strain individual variation.

It must also be noted that in all the studies discussed above, wasps were allowed uncontrolled access to hosts, in most cases for at least 24 hours. While it may be argued that such a protocol is ecologically realistic, it has the drawback of confounding a number of possible mechanisms. In particular, it is not possible from the data to discern if the relative reproductive success of the two strains is determined by the behaviour of the ovipositing female, or by some property of the individual larvae.

A possible behavioural explanation depends on both wasps laying multiple eggs in a single host. The eggs of the wasp ovipositing first hatch first. Interlarval physical combat between the siblings commences shortly after so that, by the time the eggs of the second wasp hatch most of the first wasp's offspring have eliminated each other. The larvae of the second wasp at this point outnumber the larvae from the first wasp and so it is more likely that one

of them will be the ultimate victor. Under this model, no younger larval advantage in combat is required. Collectively, the offspring of the wasp who lays the eggs that hatch last will dominate simply by arriving at the field of combat after most of the opposing larvae are already dead. Due to differences in both embryonic development time and oviposition rate (Beck *et al* 1999), there will be a greater overall lag time between a wasp being given access to hosts and her larvae hatching for the RM strain compared to the RP strain. Thus the relative reproductive success of the two strains under uncontrolled competition would be expected to show an overall bias in the RM strain's favour, and the equivalence point between the two strains to be when the RM strain is given a head start of a few hours.

An alternative explanation is that there is some systematic physiological difference between the eggs and/or larvae of the RM and RP strains that increases the probability of an RM larva winning a one on one fight with an RP larva.

To distinguish between the two explanations, it is necessary to determine the relative reproductive success of the two strains when only one egg from each strain is laid into a single host

A final issue that requires explanation is the lower reproductive success of RM wasps under intrastrain competition, compared to RP wasps. Two possibilities can be derived from the hypothesised explanations of the RM larvae's competitive advantage. If the RM larval advantage is offensive then two RM larvae may be more able to inflict mutually lethal wounds in the course of combat. However it is not obvious why this should lead to developmental failure as a pharate. A more plausible explanation is if the RM larval advantage is defensive; being mutually difficult to kill the contest between them may not be resolved until there are insufficient resources (ie the host's tissues) remaining to allow the completed development of the victor. However if this were the case the failed pharates should be relatively small while in fact they fall within the normal size range.

An intriguing third possibility is that the effect is related in some fashion to (a failure of) the RM wasp's immune system. It has been found, for a range of pathogens, that when a parasitoid grows inside an infected host

developmental failure can occur at the pupal or pharate stage, depending on the time interval between parasitism and infection (eg King and Bell, 1978; Temerak, 1980: Santiago-Alvarez and Caballero, 1990). Further, parasitism can lead to an increase in the host's resistance to a subsequent infection (Begon *et al*, 1999). If the resistance of the host is due to the effects of a parasitoid maternal secretion acting globally, and a consequence of the RM calyx tissue alteration is a general reduction in the concentration of maternal secretions, then RM-RM superparasitism may result in the victor developing in a susceptible host while for the victor of RM-RP superparasitism the host would be resistant. However, this would have to be regarded as even more speculative than most of that which precedes it.

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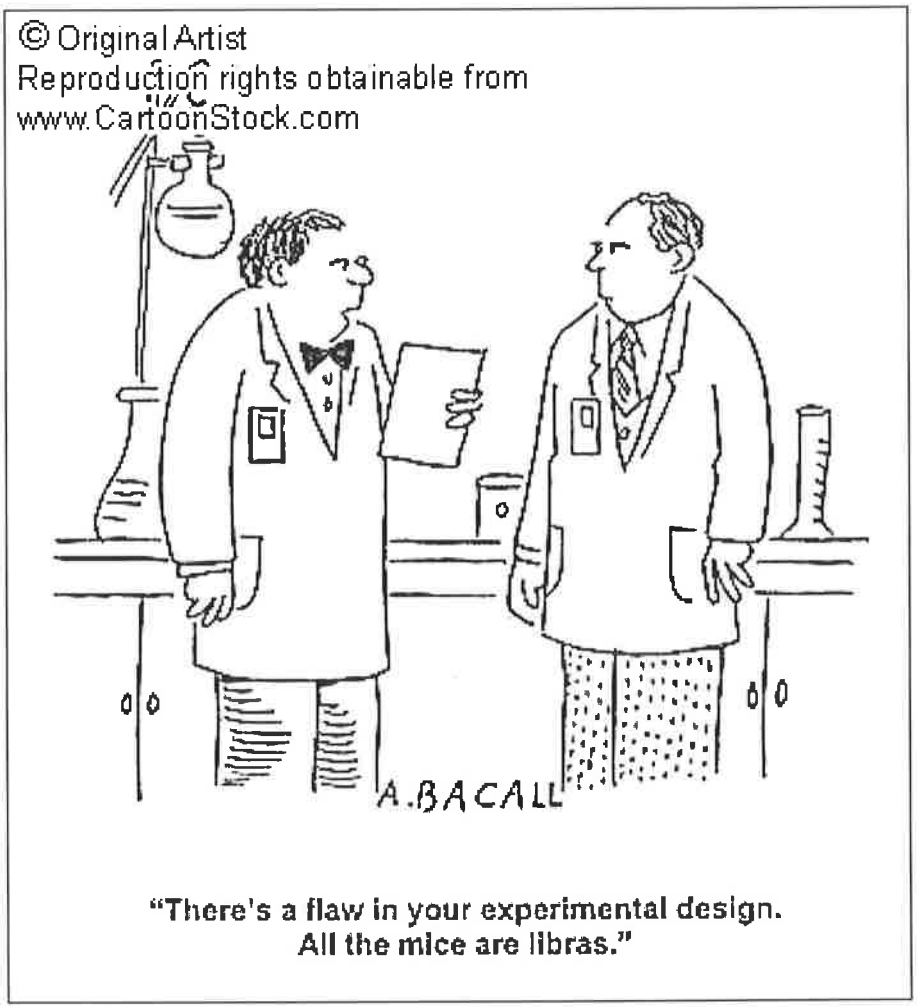
Chapter Three

Two coexisting lines of the endoparasitoid *Venturia canescens* show differences in reproductive success under con-specific superparasitism



"There's a flaw in your experimental design.
All the mice are libras."

STATEMENT OF AUTHORSHIP

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Reineke initiated the first experiment in this paper, and hence retained first authorship. However, Reineke's experimental design involved competing wasps being given uncontrolled access to hosts for various periods of time and orders of access. Roberts redesigned the first experiment as per the form in the paper, analysed and interpreted the data, conducted and analysed the additional experiments and wrote the paper.

Annette Reineke
Assisted in design and performance of experimental work, assisted in manuscript preparation and acted as communicating author.

Harry L.S. Roberts (Candidate and principal author)
Designed experiments, performed experimental work, analysed and interpreted data, wrote manuscript.

Otto Schmidt (Principal Supervisor)
Supervised work and helped in manuscript preparation.

Signed (Harry Roberts)

.....*Date*.....

Signed (Otto Schmidt)

.....*Date*.....

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Two coexisting lines of the endoparasitoid *Venturia canescens* show differences in reproductive success under conspecific superparasitism

Annette Reineke *, Harry L.S. Roberts, Otto Schmidt

Department of Applied and Molecular Ecology, The University of Adelaide, Glen Osmond, SA 5064, Australia

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Abstract

In a laboratory colony of the endoparasitic wasp *Venturia canescens* Grav. (Hymenoptera: Ichneumonidae), two genetically distinct lines (RP, RM) appear to coexist sympatrically. The two lines display pronounced differences in ovarian morphology, parasitism behaviour and number of offspring produced under competing superparasitism. Since *V. canescens* is a solitary endoparasitoid, larvae inside superparasitised hosts must compete for host possession. We examined the outcome of conspecific superparasitism between the wasp lines with different time intervals between ovipositions. The results showed that the competitive abilities of the two lines were not symmetrical. Further, the RM-line won a significantly higher fraction (around 60%) of the overall contests. Dissection of parasitoid larvae from their hosts indicated that most contests between competing larvae had occurred within the first 24 h of the eggs hatching, suggesting the advantage of the RM-line relates to physical combat. It was previously thought that the coexistence of the two lines was exclusively due to maternal effects. The results of this study indicate for the first time that these differences are based on phenotypic variations in both the larval offspring and the mother.

Keywords: Parasitoids; Superparasitism; Competition; *Venturia canescens*

1. Introduction

Most endoparasitoid wasps deposit their eggs inside the body of another species, typically an immature stage of an arthropod. The developing wasp larva selectively consumes its host, filtering nutrients from its haemolymph in a fashion that is not immediately fatal. In solitary endoparasitoids, only a single larva will be able to successfully complete its development inside a single host (Salt, 1961). However, no such limitation exists on the number of eggs that may be laid by one or more female wasps. When a host contains more than one egg, it is described as being superparasitised, a phenomenon that has been frequently observed in the field (Salt, 1934; van Alphen and Visser, 1990). Of necessity, the resulting larvae must compete for host resources, resulting in the death of all supernumerary parasitoids.

Life history theory suggests that in a variable environment, multiple evolutionary stable strategies can occur simultaneously in the same population (Stearns, 1992). For an endoparasitoid larva developing in a superparasitised host, a degree of environmental variation will be intrinsic to the system. The environment of the developing wasp larva is the host haemocoel, which will vary e.g. with the number and genotypes of the larvae present. In a superparasitised host, the task of manipulating host physiology is performed by multiple wasps, which could provide the adaptive potential for a line to divert resources toward interlarval competition and away from host manipulation. Recent studies involving a laboratory colony of the endoparasitoid wasp *Venturia canescens* Grav. (Hymenoptera: Ichneumonidae) on its host *Ephestia kuehniella* (Lepidoptera: Phycitidae) provides experimental support for this idea. Research indicates that the wasp population contains two reproductively independent lines, genotypically and phenotypically distinct, yet coexisting sympatrically (Beck et al., 1999, 2001).

* Corresponding author. Present address: Institute of Phytomedicine, University of Hohenheim, Otto-Sander-Str. 5, D-70599 Stuttgart, Germany. Tel.: +49-711-4592401; fax: +49-711-4592408.
E-mail address: areineke@uni-hohenheim.de (A. Reineke).

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In a long-standing parthenogenetic laboratory colony of *V. canescens*, two genetically distinct lines were identified on the basis of a DNA sequence variation of a gene encoding a virus-like particle (VLP1) protein (Hellers et al., 1996). The DNA coding for the protein was found to differ in the population by the presence or absence of a short tandem repeat sequence with genotypes of both clonal wasp lines accordingly being designated as 'repeat plus' (RP) or 'repeat minus' (RM). Both lines were tested for stability in the VLP1 gene over successive generations with no alterations detected in either gene. An analysis of genomic DNA using RAPD-PCR revealed a high degree of genetic variation between the two clonal wasp lines (Beck et al., 1999). Together, these results indicate that the two lines are stable and have been separated long enough to accumulate other DNA polymorphisms, and that the presence of the two VLP1 alleles in the laboratory population was not simply due to mutation pressure at this gene locus (Beck et al., 1999).

A number of phenotypic differences between the two *V. canescens* lines were also observed. Under conditions of self-superparasitism, RM wasps produced fewer offspring than RP wasps, with development of the RM pre-adults frequently failing at the pupal or pharate stage. However, under interline superparasitism, RM wasps produced significantly more offspring than RP wasps (Beck et al., 1999). Moreover, RP- and RM-females displayed behavioural differences, with RP wasps depositing eggs immediately after host encounter, while RM females showed a significant delay in parasitism. Both oviposition strategies could be correlated with two distinct ovarian phenotypes, which in turn seem to differentially influence egg maturation and egg numbers in the oviduct: RM wasps had significantly more eggs in the calyx and significantly fewer in the oviduct, and their eggs were both larger and took longer to hatch, than RP eggs (Beck et al., 1999, 2001).

Since the two wasp lines are genetically stable, and the original laboratory culture had been maintained without the addition of new stock for many years, the two lines had been coexisting sympatrically. Further, both VLP alleles have been found in all field populations of *V. canescens* analysed so far (Beck et al., 1999; Malmberg et al., 2000; Schneider et al., 2003). Homozygous RM and RP offspring from field-collected arrhenotokous wasps showed similar patterns of morphological and functional differences to the laboratory lines (Li et al., 2003), suggesting the VLP1 gene locus is genetically linked to the phenotype. However, the differences were less pronounced in the arrhenotokous females, indicating that one or more additional unlinked genes are involved in the expression of the observed phenotype.

If females accept hosts which were previously parasitised by a conspecific female, two or more parasitoid larvae have to fight for the same host. Parasitoid larvae inside superparasitised caterpillars use two mechanisms to compete for host supremacy, one involving physical fighting, the other physiological suppression with both mechanisms being dependent on the age difference of the developing parasitoid larvae: when the age difference between superparasitising larvae is only a few days, the competition for the host involves physical combat with first instar larvae possessing well-developed mouthparts biting through the cuticle of the competing larva (Fisher, 1961; Salt, 1961). The second mode of competition can occur if the lag-time between parasitism and superparasitism is around 3 days or more, and involves physiological suppression of the younger larvae by the older (Simmonds, 1943). In this situation, the younger larvae either fail to hatch or upon hatching, fail to feed or grow (Salt, 1961). In the case of *V. canescens*, the cause is most likely asphyxiation (Fisher, 1961, 1963), a consequence of the egg or young larva having a relatively high requirement for oxygen, while the older larva is both more tolerant to a low oxygen concentration and also able to consume the greater fraction of the available supply due to its larger size (Fisher, 1961, 1963).

A possible explanation of the RM-line's advantage may be based on the maternal phenotype and on both wasps laying multiple eggs in a single host, a common occurrence under laboratory culture conditions. Oviposition rates of up to 50 eggs/h, continuing until the oviducts are largely depleted of eggs, have been reported (Harvey et al., 2001), including under conditions of intra-line superparasitism (Beck et al., 2001). Regardless of the respective line, the eggs of the wasp ovipositing first hatch first. Interlarval physical combat between the siblings commences shortly after, so by the time the eggs of the second wasp hatch a number of the first wasp's offspring have already eliminated each other. The larvae of the second wasp at this point outnumber the larvae of the first wasp and so it is more likely that one of them will be the ultimate victor.

It has been previously shown that the rate of searching behaviour (Trudeau and Gordon, 1989), oviposition (Sirot et al., 1997), and the likelihood of winning an agonistic encounter (Hughes et al., 1994) all increase with egg load. RM wasps with fewer eggs in their oviducts (Beck et al., 1999) may thus tend to delay oviposition compared to RP wasps. Due to differences in both embryonic development time and oviposition rate (Beck et al., 1999), there will be a greater overall lag-time between an RM-wasp being given access to hosts and her larvae hatching compared to an RP-wasp. Thus, the relative reproductive success of the two lines when competing for hosts may show an overall bias in the RM-line's favour.

The general alternative explanation is that there is some physiological difference between the RM and RP lines that increases the probability of an RM larva winning a one-on-one encounter with an RP larva. Most plausibly, this would involve an advantage in one of the two previously identified modes of competition, either physical combat or physiological suppression.

To distinguish between the two explanations, the present study compared the reproductive success of the two lines, when one egg from each line was laid into a single host. In addition, since the age difference between competing conspecifics has been shown to affect the outcome (Marris and Casperd, 1996; Sirot, 1996), the effects of differences in the timing of oviposition were investigated. To assess the outcome of the contests, superparasitised hosts were dissected at various time intervals after oviposition. To investigate any possible differences in tolerance to a reduced oxygen environment, singly parasitised hosts were reared under atmospheric or reduced oxygen conditions and larval growth was subsequently determined.

2. Materials and methods

2.1. Insect cultures

Both hosts and parasitoids were maintained at 25 °C, under a constant light–dark regime (L14:D10) in an insect rearing room. Hosts were final instar larvae of *E. kuehniella*, reared on an oatmeal/wheatgerm/yeast diet (Harvey and Vet, 1997). Clonal RP- and RM-wasp lines from a thelytokous *V. canescens* laboratory culture were previously established as reported by Beck et al. (1999).

2.2. Competition experiments

On the day of their emergence, *V. canescens* females were removed from culture and were kept in batches of 10 with a 50% honey solution for 48 h prior to use in experiments. For the competition experiments, a single wasp from each line was put together with 20 final instar host larvae in a round plastic container (7 cm in diameter, 8 cm in height). The parasitoids were observed during oviposition and stinging attempts combined with the characteristic cocking movements of the wasp's ovipositor (Rogers, 1972) were considered as real oviposition events. Exact time of oviposition was recorded and host larvae that were parasitised once either by RP- or RM-females were immediately removed from the containers. After 4, 8, 24 and 48 h, respectively, these larvae were exposed to a wasp from the other line, wasps were again observed during oviposition and superparasitised larvae were immediately removed from the containers. These time intervals were used as they are representative of the time intervals between ovipositions that would occur under normal laboratory culture conditions. A total of 80 hosts were superparasitised in four replicates for each of the time intervals and each order of oviposition ('RP first—RM second wasp' and 'RM first—RP second wasp'). Superparasitised host larvae were cultured as described above and emerging wasps were submitted to molecular genotyping using polymerase chain reaction (PCR) to determine the number of emergent RP- and RM-wasps.

2.3. DNA preparation and molecular genetic analysis

Emerging wasps were immediately frozen and kept until molecular genetic analysis. For a crude DNA preparation, the abdomen of an individual wasp was cut off and was manually ground into small pieces with a plastic pestle in 500 μl of TES buffer (100 mM Tris–HCl, 10 mM EDTA, 2% SDS, pH 8.0). The solution was boiled for 15 min and was centrifuged for 30 s at 13,000 rpm to pellet tissue leftovers. The supernatant was transferred to a fresh tube, 0.1 vol. of 3 M NaAc and 2 vol. of ice-cold 100% EtOH were added, vortexed and incubated on ice for at least 20 min. The solution was centrifuged for 15 min at 13,000 rpm, the pellet was washed with 70% EtOH, dried and resuspended in 100 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) overnight. For PCR analysis, 3 μl of the DNA solution was used.

PCR analysis for classifying emerging wasps as RP or RM genotype followed the procedure described by Hellers et al. (1996) except that VLP1 gene specific primers DINT (5′-CTCAATATGTGGGGTGGTGG-3′) and 5′II (5′-TCGCAGTGGCTTGTCAGAGT-3′) were used amplifying a 242 and 188 bp fragment of the VLP1 gene in RP- and RM-wasps, respectively.

2.4. Size over time of larvae under interline competition

To determine at which time point after oviposition competition between larvae was resolved, 40 hosts that had been parasitised twice within 4 h or less were dissected from 4 to 8 days later and the size of the two larvae was recorded. To determine the relationship between larval age and size under the given laboratory rearing conditions, larvae were dissected from singly parasitised hosts in an additional experimental set-up. A total of 408 *V. canescens* larvae (204 from each line) were dissected out of parasitised hosts between 64 and 120 h after oviposition and their length was measured.

2.5. Larval growth under different oxygen tensions

To assess whether the two wasp lines differed in their tolerance to a reduced oxygen environment, hosts were

parasitised once by either an RM or an RP wasp and maintained for 72 h under atmospheric (21%) oxygen conditions to allow the parasitoid eggs to hatch. Parasitised hosts were then maintained for a further 120 h under either 5% or 21% oxygen, after which the parasitoid larvae were dissected out and their length measured.

2.6. Statistical analysis

All parameters recorded were analysed with the statistical software package JMP (SAS Inst. Inc. 2001, version 4.0.4). Data from the competition experiments were analysed as binary data (survival or death of the host, and relative success of superparasitism) using a generalised linear model (GLM), with time interval between ovipositions and order of ovipositions as the controlled variables. The size of larvae over time from superparasitised hosts was analysed by GLM and the relationship between age and larval size from single parasitised hosts determined by linear regression. Larval growth under atmospheric and reduced oxygen tensions was analysed by a generalised linear model, with line and oxygen tension as the controlled variables. Tukey–Kramer tests (Sokal and Rohlf, 1981) were used to conduct a posteriori comparisons; means were regarded as significantly different if $P< 0.05$.

3. Results

3.1. Competition experiments

The survival rate until parasitoid emergence and the relative proportion of offspring from the superparasitising female are shown in Table 1. Analysis by GLM revealed that the proportion of contests won by the offspring of the superparasitising female was significantly higher ($\chi^2= 12.92$, $P= 0.0003$) for the RM-compared to the RP-line. Logistic regressions of the relative pay-off from superparasitism against the time interval between ovipositions (Fig. 1) showed that when the RP-wasp oviposited second, its offspring won around 40% of contests independent of the time interval ($\chi^2< 0.01$, $P= 0.9909$). In contrast, there was a significant relation ($\chi^2= 3.95$, $P= 0.0469$) between the relative pay-off from superparasitism and the time interval between ovipositions when the RM-wasp oviposited second. When the time interval was 4 h, the RM-line won around 65% of contests; this value then declined to around 50% when the RM-wasp oviposited 48 h after the RP-wasp (Fig. 1).

Both the order of oviposition ($\chi^2= 8.82$, $P= 0.003$) and the time interval between ovipositions ($\chi^2= 27.0$, $P< 0.0001$) had a significant effect on the survival rate of superparasitised hosts; however, the interaction between these two factors was not significant ($\chi^2= 2.77$, $P= 0.0958$). Logistic regressions of the survival rate of the host against the time interval between ovipositions (Fig. 2) showed that the survival rate of the superparasitised hosts was higher when the RM-wasp oviposited second, and for both orders of oviposition decreased as the time interval increased. The latter effect is most likely due to the cost of being disturbed over a longer time interval and having to build a new burrow (Sirot, 1996).

3.2. Size over time of larvae under interline competition

All the 40 superparasitised hosts dissected were found to contain two immature larvae, thus it is unlikely that the results of the competition experiments were biased by differences between the lines in the attribution by the experimenters of oviposition success. While there was a significant positive relationship ($F= 159.2$, $P< 0.0001$) between the size and age of the larger larva from each host, there was no significant change over time ($F= 0.3592$, $P= 0.5525$) in the size of the smaller larva (Fig. 3), indicating that in the great majority of cases, the contest between competing larvae was decided within the first 24 h after hatching.

Staged dissections of larvae from singly parasitised hosts between 64 and 120 h after oviposition (Fig. 4) gave results in good agreement with previously published data on first instar *V. canescens* life history with eggs usually hatching between 66 and 74 h after oviposition (Corbet and Rotherham, 1965; Diamond, 1929). There was no significant difference in sizes of the RP and RM larvae over this time interval ($F= 0.0642$,

Table 1
Proportion of progeny surviving to emergence from 80 superparasitised hosts, and the proportion that were the offspring of the second female, for both orders of oviposition ('RP first, RM second' and 'RM first, RP second') and different time intervals between ovipositions

Time interval (h)	RM line second			RP line second		
	Proportion surviving	*N*	Proportion second females winning	Proportion surviving	*N*	Proportion second females winning
4	0.76	61	0.67	0.66	53	0.43
8	0.6	48	0.65	0.48	38	0.34
24	0.59	47	0.51	0.65	52	0.42
48	0.53	42	0.5	0.22	18	0.39

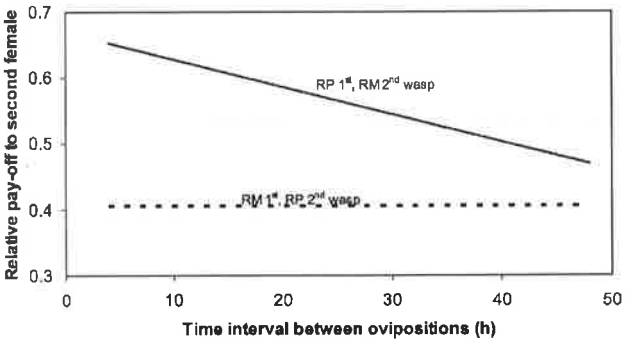

Fig. 1. The relative pay-off from superparasitism as a function of the time interval between ovipositions for the two different orders of oviposition, 'RM first, RP second' (dashed line) and 'RP first, RM second' (solid line). Lines represent the fitted values from the logistic regression model.

$P = 0.8$). Analysis by linear regression of size against age in hours ($r^2 = 0.792$, $F = 1545.6$, $P < 0.0001$) indicated that the fit was good. Inverse prediction of the age of the smaller larva from superparasitised hosts using these data indicated that, based on mean values, around 60% of contests were resolved within 6 h of the losing larva hatching and 90% within 24 h (Table 2).

3.3. Larval growth under atmospheric and reduced oxygen tensions

Under both oxygen tensions, the mean length of the RM larvae was significantly greater than the RP larvae (Table 3). Analysis by GLM revealed a significant interaction ($F = 4.09$, $P = 0.045$) between line and oxygen tension, indicative of the relative difference in size between the two lines being less under the reduced oxygen tension condition.

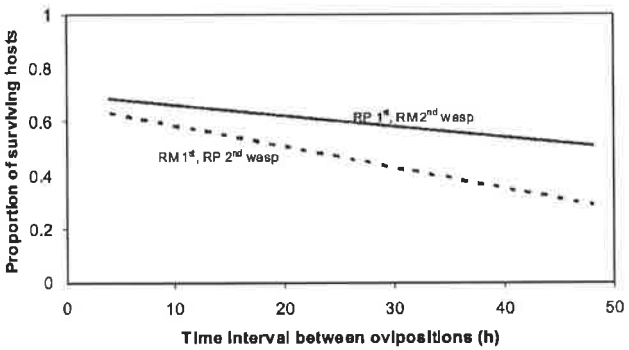

Fig. 2. Survival rate of superparasitised hosts until parasitoid emergence as a function of the time interval between ovipositions for the two different orders of oviposition, 'RM first, RP second' (dashed line) and 'RP first, RM second' (solid line). Lines represent the fitted values from the logistic regression model.

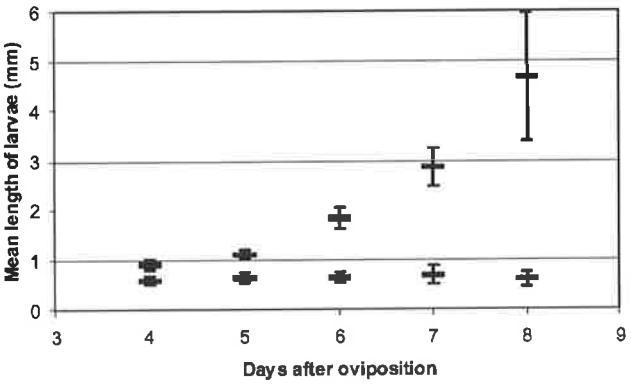

Fig. 3. Mean length of the larger and smaller larvae dissected from twice-parasitised hosts. Bars represent 95% confidence intervals of the mean.

4. Discussion

The data presented in this study indicate that the higher reproductive success of the RM line under competing superparasitism observed by Beck et al. (1999) is not due to differences in the maternal phenotype, but rather to some difference in the larval phenotype that results in an RM-advantage in one-on-one interlarval competition. The relative pay-off of superparasitism for the RP-line was around 40% independent of the time interval between ovipositions. In contrast, the relative pay-off of superparasitism for the RM-line was around 65% when the time interval between ovipositions was small, declining to 50% when the time interval was 2 days. Thus, the competitive abilities of the two lines were not symmetrical, with the RM-line winning a significantly higher fraction (around 60%) of the overall

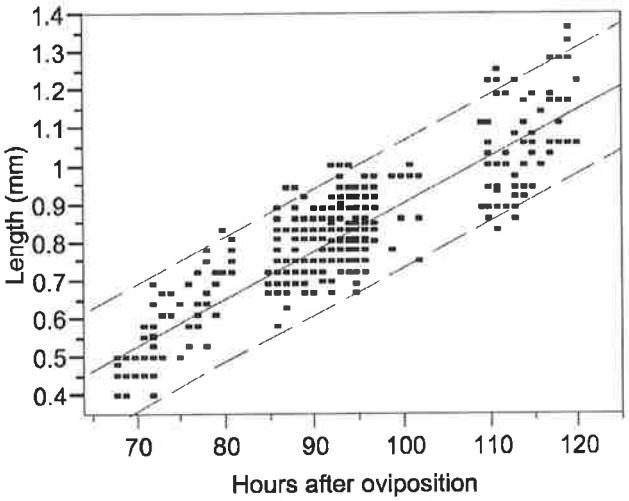

Fig. 4. Relationship between the age and length of larvae dissected from singly parasitised hosts. The solid line represents the fitted value from the logistic regression model, dashed lines represent the individual 95% confidence intervals.

Table 2
Distribution of the lengths of the smaller larvae from twice-parasitised hosts and their estimated age when the outcome of the interlarval contest was determined, by inverse prediction of the relation between age and length of larva from singly parasitised hosts

Length (mm)	Percentage of larvae	Predicted age (h)	Lower 95% CI	Upper 95% CI
0.45	7.5	64	50	77
0.5	27.5	68	54	81
0.6	27.5	76	63	89
0.7	15	84	71	97
0.8	15	92	79	105
0.9	7.5	100	87	114

contests. Furthermore, given that the RM-line has a lower rate of reproductive success than the RP-line under conditions of self-superparasitism (Beck et al., 1999), the results support the suggestion that the RM and RP phenotypes represent two evolutionary stable strategies (Maynard Smith, 1982; Stearns, 1992), under conditions of conspecific and self-superparasitism, respectively.

In addition, our data indicate that the RM-line's advantage relates to physical combat, rather than physiological suppression per se. Dissections of superparasitised hosts suggest that around 60% of contests were resolved within 6 h of the losing larva hatching and 90% within 24 h, corresponding to the time interval when physical combat has been shown to be the dominant mode of competition (Howard, 1897; Fisher, 1961). Further, although RM-larvae showed significantly higher growth rates than RP-larvae under low oxygen tension, the small size of the difference (around 12%) combined with the fact that the growth of both lines was severely retarded makes an explanation purely based on anoxic suppression implausible.

Thus, the question can be asked, what phenotypic difference can account for the RM line's advantage? The more plausible explanation is that the physiological basis for the RM advantage is due to a subtle shift in one of the factors that determines the outcome of interlarval combat, rather than through the evolution of a novel mechanism.

Table 3
Mean length of RM- and RP-larvae from singly parasitised hosts maintained first for 72 h under atmospheric (21%) oxygen conditions to allow the parasitoid eggs to hatch and then for a further 120 h under either 5% or 21% oxygen

Line	5% Oxygen				21% Oxygen			
	N	Mean length (mm)	Lower 95% CI	Upper 95% CI	*N*	Mean length (mm)	Lower 95% CI	Upper 95% CI
RM	22.00	1.37	1.28	1.46	55.00	4.20	4.06	4.34
RP	28.00	1.21	1.14	1.28	37.00	3.75	3.60	3.91

One option is that there is an interaction between physiological suppression through anoxic inhibition (Fisher, 1963) and physical combat, with the less tolerant larva being made vulnerable. Under this scenario, the RM larva would acquire the advantage through a lower absolute requirement for oxygen. However, it is unlikely that newly hatched larvae consume enough oxygen to have any affect on competitors. In addition, such a difference between the lines should lead to an increasing advantage with the age of the RM larva, which was not observed.

A second possibility is that the ovipositing RM wasp or the developing RM larva secretes a substance that is toxic to the larvae of genetically different conspecifics. Mackauer (1990) reports of such an interaction between the two closely related species, *Aphidius smithi* and *Ephedrus californicus*, with *E. californicus* wasps injecting toxic substances that, although becoming ineffective very quickly, killed *A. smithi* eggs already present in the host. However, a toxin that would affect the larvae of a conspecific *V. canescens* female after they had hatched, while not affecting self-specific progeny is difficult to envision.

A final possibility is that the outcome of interlarval combat is not determined in a simple fashion by the age difference between the two larvae, but also depends on their absolute age when the physical contest takes place. It is conceivable that the ability of a larva to successfully bite another in part depends on the degree of sclerotisation of the attackers' mandibles and of the defenders' cuticle. Thus, a physiological difference between the two lines that results in a newly hatched RM larva being more highly sclerotised would translate directly into a competitive advantage in combat over a similarly aged RP larva. Alternatively, the newly hatched RM larva may gain the advantage by being intrinsically more mobile than the RP larva. The observed higher mean growth rates of the RM larvae, possibly reflecting a higher metabolic rate, would be consistent with either possibility.

The finding that RM-larvae have a significant competitive advantage in one-on-one competition with RP-larvae confirms that the coexistence of the two lines under laboratory culture conditions is the result of a complex interaction and not simply because their phenotypic differences have no fitness consequences. In addition, it shows that the sympatric coexistence of two reproductively independent and phenotypically distinct species on the same resource is *in principal* possible. Whether this particular system occurs in natural populations of *V. canescens* is not known. While similar patterns of morphological and functional differences to the laboratory lines have been observed in field-collected wasps (Li et al., 2003), the differences were less pronounced and their contribution to the support of sympatric coexistence in the field remains to be shown.

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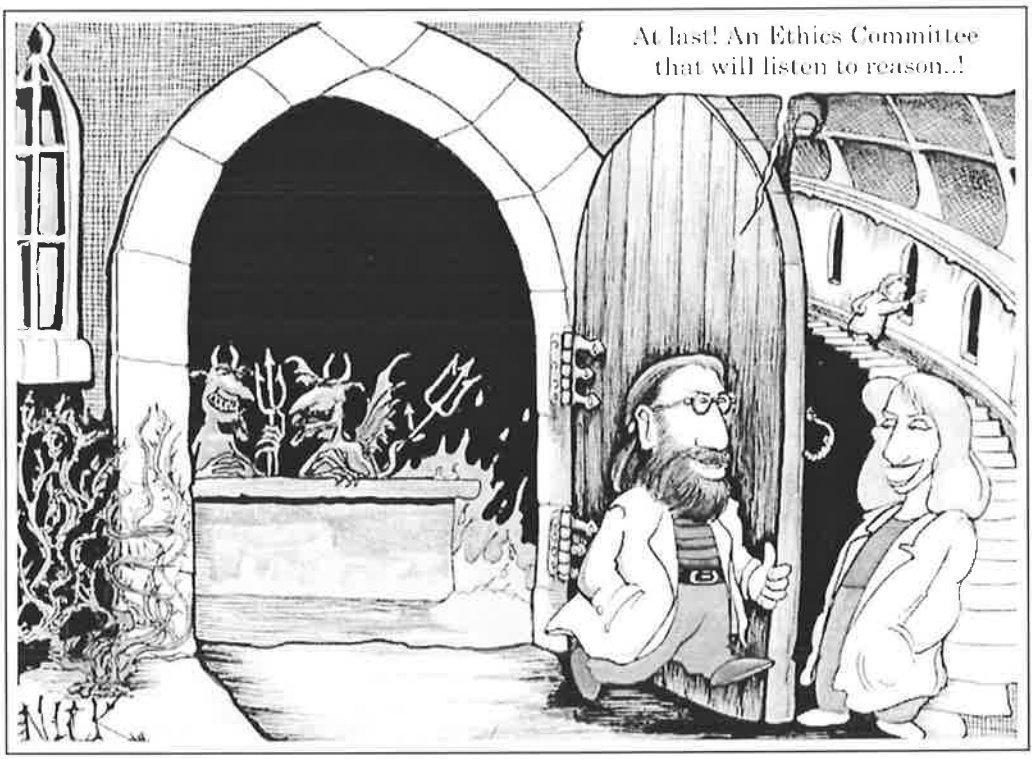
Chapter Four

The outcome of *in vitro* contests between larvae of the endoparasitoid *Venturia canescens* depends on both their relative and absolute ages



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STATEMENT OF AUTHORSHIP

Roberts, H.L.S., True, O. and Schmidt, O. (2004) The outcome of *in vitro* contests between larvae of the endoparasitoid *Venturia canescens* depends on both their relative and absolute ages. *Behavioral Ecology and Sociobiology* **57**, 132-138.

From the results of a probability tree model of the possible causes of parasitism failure (interlarval fighting, physiological suppression and host-immune response related failure) under single egg and superparasitism, Roberts concluded that the pattern of the RM-line advantage under competing superparasitism was likely to be due to the outcome of interlarval combat depending on both the relative and absolute ages of the larvae involved. To test the hypothesis, Roberts designed, performed, analysed and interpreted the experiments described, and wrote the paper. Trüe assisted in documenting the events of each taped interlarval fight.

Harry L.S. Roberts (Candidate)
Designed experiments, performed experimental work, analysed and interpreted data, wrote manuscript and acted as communicating author.

Oliver Trüe
Assisted in the performance of the experimental work.

Otto Schmidt (Principal Supervisor)
Supervised work and helped in manuscript preparation.

Signed (Harry Roberts)

.....*Date*.....

Signed (Otto Schmidt)

.....*Date*.....

Roberts, H. L. S., True, O. & Schmidt, O. (2004). The outcome of in-vitro contests between larvae of the endoparasitoid Venturia canescens depends on both their relative and absolute ages. *Behavioral Ecology and Sociobiology*, *57*(2), 132–138.

Chapter Five

The development of the endoparasitoid wasp *Venturia canescens* in superparasitised *Ephestia kuehniella*

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STATEMENT OF AUTHORSHIP

Roberts, H.L.S., Trüe, O. and Schmidt, O. (2004) The development of the endoparasitoid wasp *Venturia canescens* in superparasitised *Ephestia kuehniella*. *Journal of Insect Physiology* **50**, 839-846.

Harry L.S. Roberts (Candidate)
Designed experiments, performed experimental work, analysed and interpreted data, wrote manuscript and acted as communicating author.

Oliver Trüe
Assisted in the parasitism experiments and the determinations of host masses.

Otto Schmidt (Principal Supervisor)
Supervised work and helped in manuscript preparation.

Signed (Harry Roberts)

.....*Date*.....

Signed (Otto Schmidt)

.....*Date*.....

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The development of the endoparasitoid wasp *Venturia canescens* in superparasitised *Ephestia kuehniella*

Harry L.S. Roberts *, Oliver Trüe, Otto Schmidt

Insect Molecular Biology Laboratory, Plant and Pest Science Department, The University of Adelaide, Glen Osmond, SA 5064, Australia

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Abstract

Using a molecular marker that allows the differentiation of two strains of the solitary endoparasitoid wasp *Venturia canescens*, the study investigated the influence of host mass and the time interval between ovipositions on the survival and development of larvae from both the first and second laid eggs in superparasitised *Ephestia kuehniella*.

As the time interval between ovipositions increased both overall and superparasitism success decreased, however, time between, and order of, ovipositions had little effect on other developmental parameters. Adult size increased with host mass under both parasitism and superparasitism, while host mortality decreased with host mass under superparasitism. In addition, wasps emerging from superparasitised hosts were larger than wasps from parasitised hosts. The results confirm that for *V. canescens* on the host *E. kuehniella* both self- and conspecific-superparasitism will be an adaptive strategy when hosts are the limiting factor.

Keywords: Endoparasitoid; Life history

1. Introduction

Endoparasitoid wasps lay their eggs inside the body of a second organism, where the developing parasitoid consumes and eventually kills the host. When a host contains more parasitoids than are able to successfully complete their development, it is described as being superparasitised. This phenomenon has been frequently reported from field studies (e.g. Howard, 1897; Salt, 1934). In the case of a solitary endoparasitoid, the size of the host allows only a single larva to complete its development. If multiple eggs are present the parasitoids must compete for possession of the host, variously by ovicide, interlarval fighting or physiological suppression (Salt, 1961; Fisher, 1963; Vinson and Hegazi, 1998; Roberts et al., in press). In this situation, the first egg laid is by the parasitising female and any subsequent ovipositions are by a superparasitising female.

For many years, and despite evidence to the contrary (Hill, 1926; Salt, 1932, 1934), it was widely believed that superparasitism was due to mistakes by the ovipositioning female (Lentern et al., 1978) and that any eggs so laid were wasted (Huffaker and Matsumoto, 1982). More recently, it has been recognised that for a species where the probability of the offspring of the second female winning the competition for host possession is greater than zero, under some circumstances superparasitism can be an adaptive reproductive strategy (Hubbard et al., 1987; van Alphen and Visser, 1990).

One such species is the solitary endoparasitic wasp *Venturia canescens*. For females parasitising third instars of the moth *Ephestia kuehniella*, Sirot (1996), using a phenotypic marker to distinguish between two strains, reported that the probability of the offspring of the superparasitising female winning a competition was around 0.45 when the time interval between ovipositions was less than two days, declining to zero when the interval was four days. Since unlaid eggs can have no reproductive pay-off, this implies that conspecific-superparasitism can be adaptive under circumstances

* Corresponding author. Tel.: +61-830-37274; fax: +61-837-94095.
E-mail address: harry.roberts@adelaide.edu.au (H.L.S. Roberts).

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where hosts, rather than eggs are the limiting factor. Further, when *V. canescens* are maintained without hosts they deposit eggs onto the sides of their culture vessel whether or not their oviducts were full (Roberts and Schmidt, 2004), possibly because eggs stored in the tip of the ovipositor remain viable for only a short period. This indicates that the time frame of the decision is short. If an unparasitised host is not located within a few hours then the wasp must superparasitise or have no chance of a reproductive pay-off at all. Clearly, the optimal strategy of whether to superparasitise or to continue searching will depend on the relative reproductive values of eggs laid in already parasitised and unparasitised hosts. In part, this will depend on whether developmental parameters are affected by the parasitism status of the host.

The development of *V. canescens* under superparasitism has been studied in several different hosts. For wasps developing in the permissive host *Plodia interpunctella*, Harvey et al. (1993) found no significant differences in wasp emergence rate, development time or adult mass from third instar hosts containing one or two eggs, however, wasps developing in final instar hosts containing two eggs displayed a longer development time and a lower adult mass. In contrast, for wasps developing in final instar larvae of the semi-permissive host *Corcyra cephalonica*, when the time interval between parasitisations was a few hours superparasitism had no effect on wasp emergence rate, development time or adult mass (Harvey et al., 1996). This indicates that the parasitism status of the host can affect the reproductive value of an offspring, in a fashion that varies between host species.

However, in these studies the effects of the order of, and the time between, parasitisations on developmental parameters were not investigated, due to difficulties in determining whether an emergent parasitoid is the offspring of the first or second female to oviposit. By taking advantage of the recent discovery of a molecular marker that allows the differentiation of two strains of *V. canescens* (Hellers et al., 1996; Beck et al., 1999), the present study extends the work of Harvey et al. (1993, 1996) by investigating the influence of host mass and the time interval between ovipositions on the development of larvae from both the first and second laid eggs. By examining pre-adult development in a different host (*E. kuehniella*), the study also provides an additional cross host species comparison of the development of *V. canescens* under superparasitism.

2. Methods

The wasps were clonal RP- and RM-lines of *V. canescens* initially established by Beck et al. (1999). Prior to the described experiments, cultures of the two lines were maintained for a number of generations under conditions of predominantly single egg parasitism. It was subsequently observed that the phenotype of the new RM-line culture (now called RMN) was significantly different in a number of characters from the old RM-line culture (now called RMO). Whether the observed differences between the RMN- and RMO-lines are a consequence of the alterations in the culture conditions or due to contamination is unclear and is currently being investigated.

The wasps were reared in cylindrical clear plastic tubs (height 20 cm and diameter 15 cm). Three or four adult wasps were placed into each container with 40–50 hosts. Upon emergence, the wasps were removed from culture and were kept in gauze-covered 425 ml clear plastic cups (Party Rite Jumbo Tumblers®, Harris Paper Pty. Ltd., West Heidelberg, Australia) with a 50% honey solution. Wasps used in the experiments were 5–10 days old. Hosts were *E. kuehniella*, reared on a 10:2:1 mixture of oat bran, wheat germ, and dried brewers yeast. All experiments were conducted and cultures maintained at $25 \pm 1\ ^{\circ}C$, under a constant light–dark regime (L14:D10).

To obtain singly parasitised hosts, a single wasp was put together with 25–30 host larvae of varying sizes in a plastic container (7 cm in diameter, 8 cm high). The parasitoids were observed during oviposition and stinging attempts that resulted in a startle response from the larvae, combined with the characteristic cocking movements of the wasp's ovipositor (Rogers, 1972) were considered as real oviposition events. Any stinging attempt that either did not evoke a startle response or was not followed by a cocking movement was regarded as uncertain and the larva was discarded. After being parasitised, the hosts were weighed and maintained individually in glass vials with excess food until parasitoid eclosion. Over 60 hosts were singly parasitised by each line.

To obtain superparasitised hosts, *E. kuehniella* larvae that had been parasitised once were presented to a wasp of the other line 4, 8, 24, 48 and 72 h later, respectively. Wasps were again observed during oviposition, and superparasitised larvae were immediately removed from the container as above. At least 50 hosts were superparasitised for each of the time intervals and each order of oviposition.

Emergent wasps were killed by freezing and the time and date of emergence recorded, which allowed development time to be calculated following molecular genotyping to determine the line of the wasp. Head capsule width was measured to the nearest 0.0125 mm with an optical micrometer.

Hosts from which a wasp had not emerged by day 35 were dissected to determine whether developmental failure had occurred as an early instar (dry host mummy), a late instar or pupa (dead larva/pupa ident-

ifiable in host) or a pharate (some part of the developing adult cuticle visible).

Molecular genetic analysis was performed following the method of Reineke et al. (2004).

2.1. Statistical analysis

Data were analysed using the generalised linear model (GLM) platform, JMP V4.0.4 (SAS 2001), with continuous factors centred by their means (Neter et al., 1990). Analyses started with full models with all interactions, and non-significant interactions were progressively dropped.

3. Results

3.1. Development in singly parasitised hosts

Of the 222 hosts parasitised, two produced moths while a further 28 died during larval development. Analysis revealed that neither strain nor host mass had a significant effect on parasitism success, (offspring from hosts: RP = 87.5%, RMN = 86.4%). An analysis of the stage at which developmental failure occurred revealed that in over 80% of cases failure occurred when the wasp was a late instar or pharate (Fig. 1), with the effects of neither strain nor host mass being significant.

Data from a further 58 wasps, from trials in which it was not clear that oviposition had occurred, were included in the analyses involving developmental parameters. Analysis of wasp development time with strain and host mass as factors revealed a significant but weak positive relationship ($F = 22.4$, df $= 1,233$, $p < 0.0001$, $R^2 = 0.063$) between host mass and development time (mean development time for offspring from 10 mg hosts = 23.4 d, from 45 mg hosts = 24.1 d) as well as a small but significant difference between the strains ($F = 13.0$, df $= 1,233$, $p = 0.0004$; least squares means: RMN = 23.6 d, RP = 23.9 d).

Analysis of wasp head capsule width with strain, host mass and development time as factors revealed a significant positive relationship ($F = 210.1$, df $= 1,231$, $p < 0.0001$) between host mass and wasp size (Fig. 2a), a small but significant difference between the strains ($F = 14.1$, df $= 1,231$, $p = 0.0002$; least squares means: RMN = 1.27 mm, RP = 1.25 mm) and a significant positive relationship ($F = 15.4$, df $= 1,231$, $p < 0.0001$) between development time and wasp size (Fig. 3).

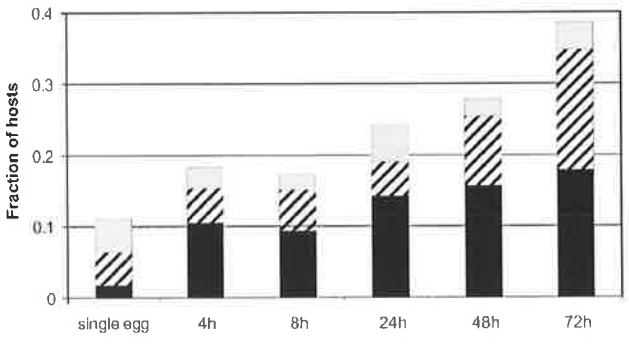

Fig. 1. Stage of *V. canescens* larva when developmental failure occurred, as a fraction of total hosts (*E. kuehniella*) under single parasitism, and superparasitism with different time intervals between oviposition. Black represents early larva, diagonal bars represents late larva/pupa and grey represents pharate adult.

3.2. Development in superparasitised hosts

Of the 593 hosts superparasitised, only six produced moths while a further 141 died during larval development. Analysis of relative superparasitism success with order of ovipositions, time interval between ovipositions and host mass as factors revealed that only the time interval between ovipositions ($\chi^2 = 16.7$, df $= 1,438$, $p < 0.0001$) had a significant effect, with the probability of relative superparasitism success

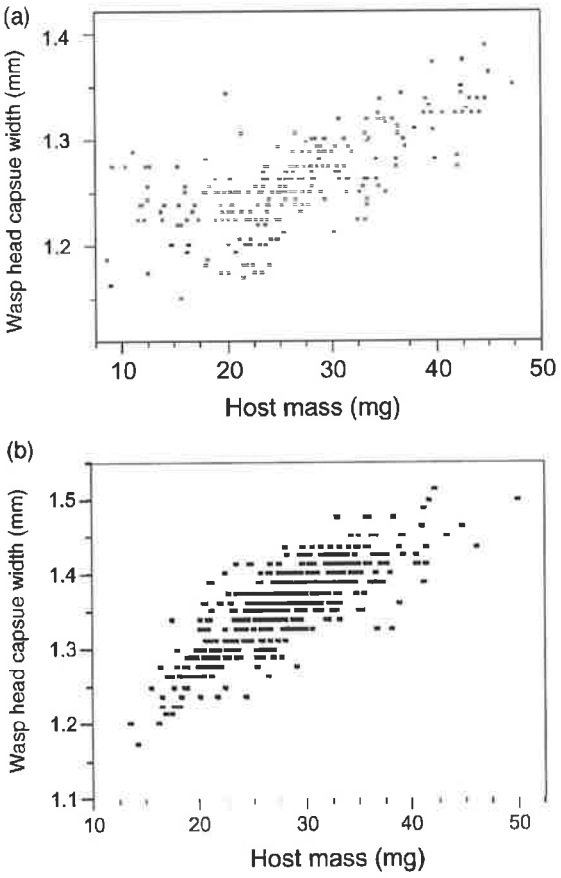

Fig. 2. The relationship between the mass of the host (*E. kuehniella*) and the head capsule width of the emergent *V. canescens* under (a) single parasitism and (b) superparasitism.

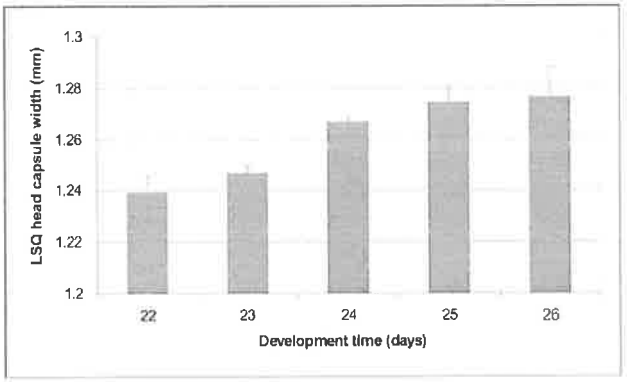

Fig. 3. Relationship between development time of *V. canescens* in *E. kuehniella* under single parasitism and least squares mean adult head capsule width of *V. canescens*. Bars represent one standard error of the mean.

decreasing as the time interval between ovipositions increased (Fig. 4a). A similar analysis on survival or death of the host similarly revealed that host mortality increased significantly ($\chi^2 = 13.6$, df $= 1,589$, $p = 0.0002$) with the time interval between ovipositions (Fig. 4b), and also decreased significantly ($\chi^2 = 12.4$, df $= 1,589$, $p = 0.0004$) with increasing host mass (Fig. 4c), while the effect of order of ovipositions was not significant. Analysis of the stage at which developmental failure occurred, relative to the number of hosts that failed, with time between ovipositions and host mass as factors, revealed no significant differences, with failure occurring as early instars in over 50% of cases (Fig. 1).

Analysis of wasp development time, with order of ovipositions, time interval between ovipositions, strain of emergent wasp and host mass as factors revealed a significant positive relationship between time interval between ovipositions ($F = 23.95$, df $= 1,432$, $p < 0.0001$) and wasp development time (Fig. 5), as well as a weak but significant relationship between host mass ($F = 5.07$, df $= 1,432$, $p = 0.0248$, $R^2 = 0.009$) and development time. There was also a significant interaction between order of ovipositions and strain of emergent wasp ($F = 8.25$, df $= 1,432$, $p = 0.0043$), suggesting that the offspring of the second female to oviposit showed longer development times. This was confirmed by a second analysis (development times least square means: offspring of first female = 23.8 d, offspring of second female = 24.2 d; $F = 12.1$, df $= 1,432$, $p = 0.0005$). The effects of order of ovipositions and strain of emergent wasp were not significant.

Analysis of wasp head capsule width, with order of ovipositions, time interval between ovipositions, strain of emergent wasp and host mass as factors revealed a significant positive relationship ($F = 825.7$, df $= 1,437$, $p < 0.0001$) between host mass and wasp size (Fig. 2b),

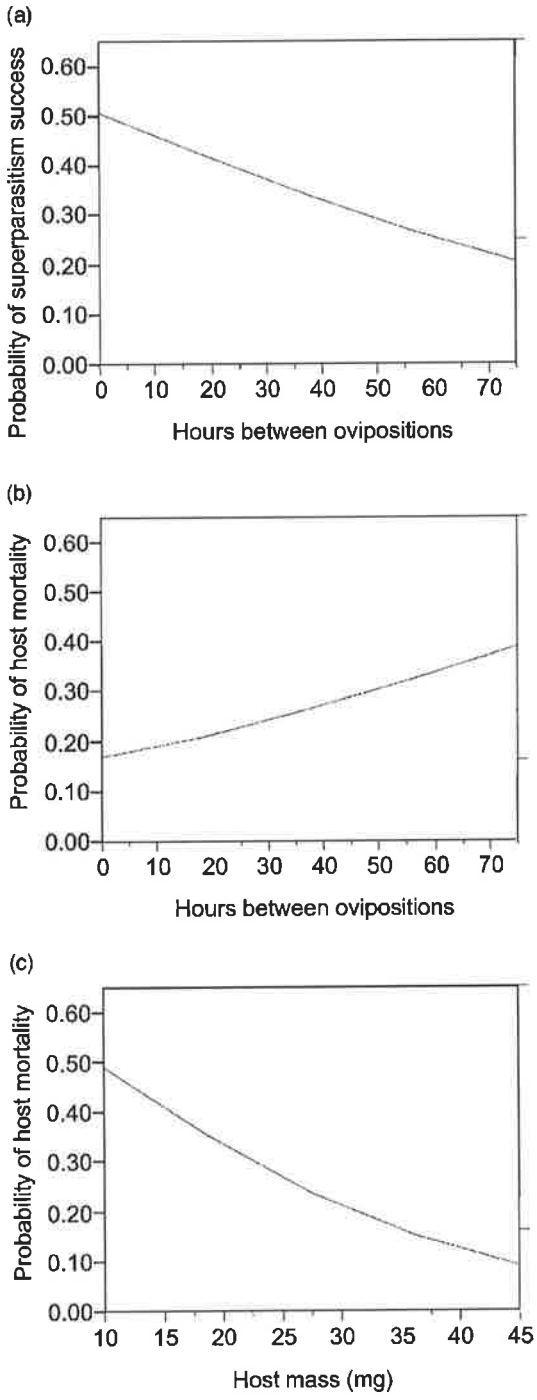

Fig. 4. Logistic regressions on survivorship data. Regression lines are based on the outcome from 593 superparasitised hosts ranging in mass from 10 to 45 mg (individual data points not shown). (a) The time interval between ovipositions and the probability that the emergent *V. canescens* was the offspring of the superparasitising female. Note that hosts from which no *V. canescens* emerged ($N = 147$) were excluded from this analysis. (b) The time interval between ovipositions and the probability that the superparasitised *E. kuehniella* died before the emergence of *V. canescens*. (c) The mass *E. kuehniella* and the probability that the superparasitised host died before *V. canescens* emergence.

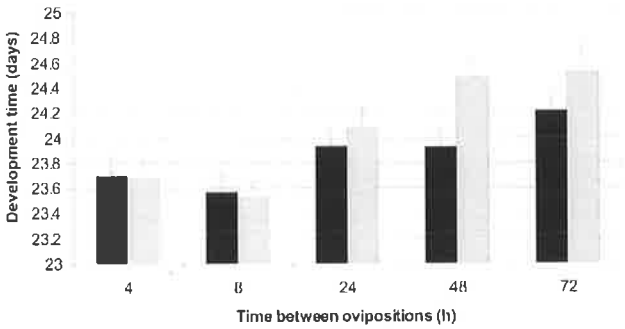

Fig. 5. Effect of time interval between ovipositions on mean development time of *V. canescens* larvae in superparasitised *E. kuehniella*. Black represents offspring of the parasitising female, grey represents offspring of the superparasitising female. Bars represent one standard error of the mean.

a small but significant difference between the strains ($F = 30.0$, df $= 1,437$, $p < 0.0001$; least squares means: RM = 54.5, RP = 53.9) and a significant interaction between strain of emergent wasp and host mass ($F = 7.08$, df $= 1,437$, $p = 0.0081$). The effects of order of, and time interval between, ovipositions were not significant.

3.3. Comparison of development in singly versus superparasitised hosts

To compare the effects of single parasitism versus superparasitism, only the superparasitism data for a time interval between ovipositions of 4–8 h were used.

Analysis of survival or death of the host with number of eggs and host mass as factors revealed that significantly fewer wasps emerged from hosts containing two eggs ($\chi^2 = 4.037$, df $= 1,457$, $p = 0.0445$), while the effect of host mass was not significant. Analysis of the developmental stage at which failure occurred revealed significant differences ($\chi^2 = 11.79$, df $= 2,63$, $p = 0.0028$) between one and two egg hosts, with over 55% of failures occurring as early instars in superparasitised hosts compared to less than 17% in single parasitised hosts. In terms of the fraction of total hosts (Fig. 1), single parasitised hosts failed as early instars in 1.85% of cases while superparasitised hosts failed as early instars in 9.88% of cases.

Analysis of wasp head capsule width, with strain of the emergent wasp, number of eggs and host mass as factors revealed that in addition to the significant effects of strain ($F = 13.06$, df $= 1,435$, $p = 0.0003$) and host mass ($F = 469.3$, df $= 1,435$, $p < 0.0001$), wasps emerging from one egg hosts were significantly smaller than those emerging from two egg hosts ($F = 627.4$, df $= 1,435$, $p < 0.0001$; least squares means: one egg = 1.25, two egg = 1.34). There was also a significant interaction between number of eggs and host size ($F = 30.19$, df $= 1,385$, $p < 0.0001$), indicative of the difference between one and two egg hosts increasing with host size (Fig. 2a and b).

A similar analysis of development time revealed no significant differences between one and two egg hosts (mean development time in days: one egg host = 23.77; two egg host, first ovipositing female = 23.63, second ovipositing female = 23.88). Mirroring the results for the individual treatments, the effects of host mass ($F = 20.3$, df $= 1,426$, $p < 0.0001$) and strain of the emergent wasp ($F = 21.4$, df $= 1,426$, $p < 0.0001$) were significant.

4. Discussion

4.1. The effects of time interval between ovipositions

Consistent with the results of Sirot (1996), the value of a host to the superparasitising female decreased with the time interval between ovipositions, with an increase in host mortality (Fig. 4b) and a decrease in relative superparasitism success (Fig. 4a) with increasing time interval. Dissection of failed hosts revealed increases with time interval in mortality at both the early and late larval stages (Fig. 1), suggesting the increase in host mortality is due to a general stress effect, either from being physically disturbed (Sirot, 1996) or a response to the maternal secretions. In addition, there was a small increase in development time (Fig. 5), but no significant relationship was observed between adult wasp size and time interval between ovipositions. This is consistent with previous observations that interlarval contests are resolved at the latest within a few hours of the second larva hatching (e.g. Fisher, 1961; Reineke et al., 2004), and thus before either larva has consumed a significant quantity of the host's tissues.

4.2. The effects of order of ovipositions

The order of ovipositions had only a small effect on development, with the offspring of the second female to oviposit showing a significant increase in development time. As the order of ovipositions had no significant effect on the size of the emergent adult, this effect may represent inhibition of embryonic or early first instar development, due to the first hatching larva selectively removing key nutrients. However, while statistically significant the effect was small (<2%) and the significance of this for parasitoid fitness is uncertain.

4.3. The effects of hosts mass

Looking at the effects of host size on development, under both single egg and superparasitism adult size increased substantially with host mass (Fig. 2a and b). Although host instar was not determined, the range of

host masses (10–50 mg) indicates that hosts ranged from third to fifth instars. A significant increase in pre-adult development time with host mass was also observed for both conditions, but the effect was small (<5% between hosts of mass 10–45 mg) and the significance of this for parasitoid fitness is uncertain. No significant change in the rate of adults from hosts with increasing host mass was observed under single parasitism. Under superparasitism, host mortality was higher for smaller hosts, however, dissection of failed hosts found that the relative fractions of the stages at which failure occurred did not vary with host size. This suggests the higher host mortality in smaller hosts is again due to a general stress effect such as smaller hosts being at more adversely affected by the increased amount of venom (Harvey et al., 1993) or the consequences of parasitoid combat (Vinson and Sroka, 1978).

In addition, a weak positive relationship was observed between pre-adult development time and adult size (Fig. 3), suggesting a possible trade off between parasitoid developmental speed and the reproductive output of the emergent female.

4.4. Comparison of parasitism and superparasitism

Comparing the development of *Venturia* in single parasitised and superparasitised *Ephestia* revealed that superparasitism resulted in a lower rate of emergent adults from hosts. This is probably due to the fraction of interlarval contests in which both parasitoids die. Dissection of failed hosts indicated that the increase in mortality was primarily due to an 8% increase in the total fraction of hosts that failed as early instars (Fig. 1, columns 'single egg' and '4 h'), a value similar to the fraction (10%) of interlarval contests under in vitro conditions that resulted in mutual death (Roberts et al., in press). More interestingly, the size of the emergent wasps was found to be greater under superparasitism, with the size of the difference increasing with host size (Fig. 2a and b). It has been shown that for *V. canescens* lifetime egg production increases with adult size (Harvey et al., 2001; Roberts and Schmidt, 2004). Based on size and fecundity data for RP-line females (Roberts and Schmidt, 2004), it can be calculated that the mean fecundity for an RP-female developing in a 40 mg fifth instar host increases from 500 eggs under single egg parasitism to 620 eggs under superparasitism, or an increase of around 25%. Increases in adult size under superparasitism have also been reported for the aphidid wasp *Aphidius ervi* (Bai and Mackauer, 1992) and the braconid wasp *Monoctonus paulensis* (Mackauer and Chau, 2001). An implication of this finding is that, in addition to acting as an insurance policy against the risk of future conspecific-superparasitism, self-superparasitism of *E. kuehniella* by *V. canescens* can have a positive pay-off when hosts are the limiting factor (Mackauer et al., 1992).

4.5. Development in P. interpunctella and E. kuehniella

A comparison of the development of *V. canescens* in third and fifth instars of the two hosts *P. interpunctella* (Harvey et al., 1993) and *E. kuehniella* revealed substantial differences (Table 1). Most strikingly, the rate of host mortality under superparasitism was lower for fifth instar *Ephestia* but higher for fifth instar *Plodia*, while adult size increased from third to fifth instars and with number of eggs in *Ephestia* but variously decreased or was unchanged in *Plodia*. The patterns of developmental changes under superparasitism in these hosts are also different from those observed in *C. cephalonica* (Harvey et al., 1996), and indicates that the conditions under which superparasitism is adaptive will be specific to the individual parasitoid–host system.

4.6. How does V. canescens manipulate host physiology?

It has been shown for a number of parasitoid species that proteins from the venom gland and those produced by polydnaviruses play roles in manipulating both host immune responses and metabolic functions (e.g. Beckage, 1998). There is also evidence that the larvae of a number of species secrete metabolically active substances such as ecdysteroids (Brown et al., 1993) and mucopolysaccharides (Führer, 1973) into the hosts' haemolymph. The results of this study support the suggestion by Harvey (1996) that *V. canescens* also employs secretions from both the ovipositing female and the developing larva to actively modify the host larva's physiology. When a host is superparasitised and, as a consequence of interlarval combat both para-

Table 1
Comparison of the effects of host size (as L3 and L5 instar) and number of eggs per host, on the development of *V. canescens* in *Plodia interpunctella* (Harvey et al., 1993) and *Ephestia kuehniella*

	L3 vs L5, one egg		L3 vs L5, two eggs		L3, one vs two eggs		L5, one vs two eggs	
	Plodia	*Ephestia*	*Plodia*	*Ephestia*	*Plodia*	*Ephestia*	*Plodia*	*Ephestia*
Development time	L3 = L5	L3 < L5	L3 < L5	L3 < L5	1 = 2	1 = 2	1 < 2	1 = 2
Adult size	L3 = L5	L3 < L5	L3 > L5	L3 < L5	1 = 2	1 < 2	1 > 2	1 < 2
Adults from hosts	L3 > L5	L3 = L5	L3 > L5	L3 < L5	1 > 2	1 > 2	1 > 2	1 > 2

sitoids die, the host almost invariably also dies. In contrast, a host parasitised numerous times that, following the resolution of interlarval contests, contains one viable parasitoid and many dead ones will in most cases give rise to an emergent wasp. In the first case, if maternal secretions were not affecting the hosts' physiology then the death of the parasitoids should allow the no-longer-parasitised host to complete its development. It is implausible that host failure in this situation is due simply to the two decomposing parasitoid carcases since multiparasitised hosts, containing numerous dead parasitoids, generally produce a wasp. Under these circumstances the potentially fatal consequences of the putative secretions are prevented by the presence of a viable larva, which suggests that the developing parasitoid, either by selectively filtering components from the host haemolymph or by secretion of metabolically active substances, is also altering the hosts' physiology. In addition, since the maternal secretions of *V. canescens* do not include polydnaviruses, the effects of any secretions are likely to be concentration dependent. If so, then the effects should be greater under superparasitism, where two aliquots of maternal secretions are injected into the host, and as a consequence of dilution, developmental differences between single and superparasitised hosts should increase with host size.

The results of Corbet (1968) are consistent with both of these propositions. In one experiment, final instar *E. kuehniella* were parasitised by *V. canescens*, and after three days eggs were dissected from the hosts and injected into fresh hosts that, therefore, did not contain maternal secretions. It was found that the subsequent development of the transplanted parasitoids was significantly retarded compared to non-transplanted controls. While this may simply reflect the effects of the injection process, it is also consistent with maternal secretions altering host metabolism. A second experiment examined the effects of parasitism on the freezing point depression of host haemolymph, as an indicator of solute concentration, under a number of treatment conditions. It was observed that parasitism resulted in an increase in freezing point depression compared to unparasitised hosts, but a reduction in freezing point depression compared to controls under treatments (abdominal ligation and decerebration) that otherwise led to an increase in freezing point depression. Further, in the latter case the reduction was not observed in hosts three days after parasitisation, but only after the larvae had hatched. While it is unclear from this whether the effect is due to larval secretions or simply larval feeding, the results indicate that in this situation it is the young developing parasitoid that is altering the physiology of its host.

The available evidence indicates that *V. canescens* larvae do not feed directly on host tissues but rather by filtering soluble components from the hosts' haemolymph. Thus, the haemolymph can be viewed as a nutrient sink that is replenished from the fat body as its concentration is reduced by larval feeding. In this context, it is tempting to speculate that one of the roles of the maternal secretions is to inhibit host control of this process, while the secretions of the larva manipulate the process to facilitate its own development. Under superparasitism the inhibition by maternal secretions is more pronounced, leading to the higher failure rate observed in smaller larvae, and the later larval manipulation more effective, leading to the increasing difference observed in adult size (Fig. 2a and b). Further experiments are required to clarify this issue.

In summary, the study found that as the time interval between ovipositions increased both overall and superparasitism success decreased, while there was little effect on other developmental parameters. Adult size increased with host mass under both single parasitism and superparasitism, while host mortality decreased with host mass under superparasitism. In addition, wasps emerging from superparasitised hosts were larger than wasps from single parasitised hosts. The results confirm that for *V. canescens* developing in the host *E. kuehniella* both self- and conspecific-superparasitism will be an adaptive strategy when hosts are the limiting factor. Speculatively, the results also suggest that the metabolic activity of the host is actively modified initially by secretions of the ovipositing female and later, by the parasitoid larva as it develops inside the host.

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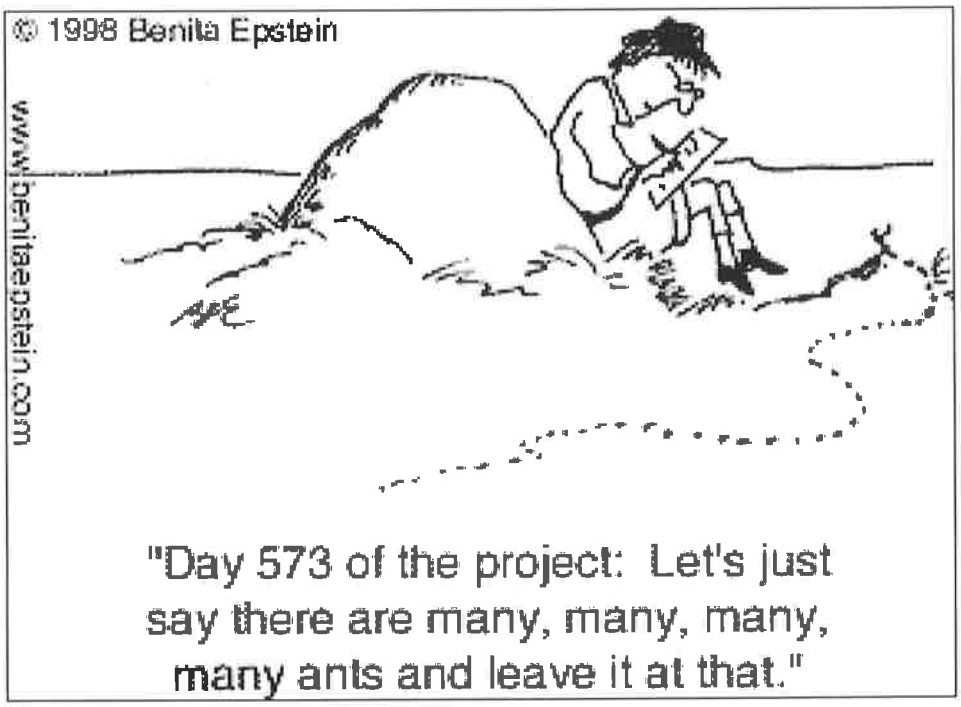
Chapter Six

Lifetime egg maturation and deposition by host-deprived *Venturia canescens*



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Harry L.S. Roberts (Candidate)
Designed experiments, performed experimental work, analysed and interpreted data, wrote manuscript and acted as communicating author.

Otto Schmidt (Principal Supervisor)
Supervised work and helped in manuscript preparation.

Signed (Harry Roberts)

.....*Date*.....

Signed (Otto Schmidt)

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Lifetime egg maturation by host-deprived *Venturia canescens*

Harry L.S. Roberts*, Otto Schmidt

Insect Molecular Biology Laboratory, Plant and Pest Science, The University of Adelaide, Glen Osmond, SA 5064, Australia

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Abstract

The study investigated egg maturation and deposition by the endoparasitoid wasp *Venturia canescens* under conditions of host deprivation. Female *V. canescens* maintained without hosts began to deposit eggs onto the sides of the culture vessel on the day of eclosion. The maturation of additional eggs was not inhibited once the maximum oviduct egg load was reached but rather continued for the duration of the experiment (up to 39 days), at a rate of around 5.8% of the remaining unmatured eggs per day. Following host access, wasps matured additional eggs at an increased rate. Artificial damage to the ovipositor resulted in a reduced rate of egg maturation even though the oviducts were partly egg depleted, while damage to the auxiliary valvulae had no effect. These results suggest two conclusions. Under conditions of host deprivation, the rate at which eggs are matured is determined by the rate of synthesis of precursors by the fat body that in turn is modified by feedback from the ovipositor, induced by physical stimulation. Further, the discarding of eggs is due to the involuntary unidirectional movement of eggs down the oviduct, facilitated by the ongoing maturation of additional eggs.

Keywords: Endoparasitoid; Synovigenic; Hydropic; Egg load

1. Introduction

Parasitoid wasps deposit their eggs either outside (ectoparasitoid) or inside (endoparasitoid) the body of their host. After hatching, the immature parasitoid consumes and ultimately kills its host. Since suitable hosts are unlikely to be continuously available, the extent to which the female parasitoid can match egg production to host availability will significantly affect her lifetime reproductive success (Flanders, 1950; Jervis et al., 2001). To this end, a number of egg production strategies exist. The eggs may be small and deficient in yolk (hydropic), capable of being produced without the female having access to an extrinsic protein source, or large and yolk-rich (anhydropic), with continued production being dependent on the female being able to feed on an extrinsic protein source such as host haemolymph (Flanders, 1942; Jervis and Kidd, 1986). In addition, Flanders (1950) argued for a distinction between parasitoids that emerge with a full complement of mature eggs (pro-ovigenic) and those that continue to mature additional eggs after emergence (synovigenic). This dichotomy was extended by Jervis et al. (2001), who showed that the "ovigeny index" of a wasp (the fraction of the maximum potential lifetime complement of eggs that are mature upon emergence) occurs as a continuum across species and is negatively correlated with lifespan. Within a species, it has been suggested that the ovigeny index of an individual would decrease with increasing body size (Jervis et al., 2003). Among anhydropic egg producers, those species that have the capability to resorb eggs tend to have a lower ovigeny index, that is, they mature a greater proportion of their eggs after emergence.

The solitary endoparasitoid *Venturia canescens* Grav. (Hymenoptera: Ichneumonidae) is strongly synovigenic (has a low ovigeny index), and produces hydropic eggs. At emergence, the oviducts of the adult wasp contain between 20 and 60 mature eggs, and in the first day, additional eggs are matured at a rate of approximately 1.8 eggs per hour (Trudeau and Gordon, 1989; Fletcher et al., 1994). If the wasp is deprived of hosts,

* Corresponding author. Tel.: +61-83037274; fax: +61-83794095.
E-mail address: harry.roberts@adelaide.edu.au (H.L.S. Roberts).

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then the eggs accumulate in the oviducts. Harvey et al. (2001) reported that oviduct egg numbers continue to increase for several days before attaining a maximum of about 160 eggs after 6 days, after which egg numbers remain constant. This suggests that when the egg storage capacity of the oviducts has been reached, oogenesis ceases. However, it was recently observed that host-deprived *V. canescens* deposit substantial numbers of eggs on the sides of the containers in which they are maintained (Roberts and Zengqi, pers. obs.). While some anhydropic egg-producing females will deposit partially resorbed eggs if host-deprived (e.g. *Phaeogenes nigridens*, Smith, 1932), cases of hydropic egg-producing endoparasitoids depositing eggs when hosts are unavailable have not been previously reported.

The observation raises two questions: why does the female deposit eggs in the absence of hosts and what controls the rate of oogenesis? Egg deposition in the absence of hosts may have a behavioural or physiological basis. During host seeking, an egg is stored in the tip of the ovipositor, ready to be laid should a host be encountered. Thus, possible behavioural explanations include accidental deposition during host seeking or the deliberate discarding of degenerate mature eggs. An alternative physiological basis could be, if egg production cannot be completely inhibited, then once the oviducts are full, any surplus would have to be discarded. Similarly, if the movement of eggs down the oviducts cannot be prevented, then eggs may be inadvertently forced out.

The rate at which eggs are matured by host-deprived wasps may be influenced by feedback on oviduct egg numbers or egg deposition behaviour, or alternatively it may be independent of any feedback and determined solely by metabolic processes.

At the metabolic level, the process of oogenesis in anhydropic egg producing insects generally is known to be controlled hormonally at a number of different points, including the separation of follicles from the germ cells, the previtellogenic growth of the oocyte, the synthesis of precursor molecules, and their uptake by the oocyte (de Wilde and de Loof, 1973). While very little is known about the endocrine control of oogenesis in hydropic egg producers (Wheeler, 1996), in broad terms, the possible control points must be the separation of follicles from the germ cells, the synthesis of precursor molecules by the fat body and their utilisation by the follicles.

Here, we document the previously unreported phenomenon of egg deposition by a host-deprived endoparasitoid wasp. We provide evidence that the rate at which *V. canescens* matures eggs is determined by the rate of synthesis of precursor molecules by the fat body, which in turn is modified by sensory feedback from the ovipositor. Further, the data suggest that the discarding of eggs is due to the involuntary unidirectional movement of eggs down the oviduct, which is facilitated by the ongoing maturation of additional eggs.

2. General methods

All experiments were conducted and cultures maintained at 25 ± 1 °C, under a constant light–dark regime (L14:D10).

2.1. Insects

The wasps were a clonal repeat plus (RP) line from a thelytokous *V. canescens* laboratory culture previously established as reported by Beck et al. (1999). The wasps were reared in cylindrical clear plastic tubs: height 20 cm and diameter 15 cm. Three or four adult wasps were placed into each container with 40–50 hosts. Upon emergence, the wasps were removed from culture and were kept in gauze-covered 425 ml clear plastic cup (Party Rite Jumbo Tumblers™, Harris Paper Pty. Ltd., West Heidelberg, Australia) with a 50% honey solution. Hosts were final instar *Ephestia kuehniella*, reared on a 10:2:1 mixture of oat bran, wheat germ, and dried brewers yeast.

2.2. Correlation between physical parameters

In *V. canescens*, egg load has been shown to increase with wasp size (Harvey et al., 1994). The aim of this experiment was to determine the physical parameter that best correlated with wasp dry mass. Within 2 h of emergence, wasps were killed by freezing and then dried at 50 °C for 24 h. Following this, they were weighed to the nearest 0.0001 g, and the front wing, rear tibia and head capsule width were measured under a stereomicroscope.

2.3. Measurement of oviduct and deposited egg numbers

Wasps were individually maintained in a 425 ml clear plastic cup (Party Rite Jumbo Tumblers™, Harris Paper Pty. Ltd., West Heidelberg, Australia) sealed with gauze. A small weighing tray containing a 50% honey solution soaked into cotton wool was placed in the bottom of the cup as a sugar source. Wasps were dissected at times ranging between 0 and 39 days after eclosion (Table 1). To determine egg numbers, wasps were chilled on ice and dissected in water under a stereomicroscope. The width of the head capsule was measured and the number of eggs present in the calyx region and the lateral oviducts were counted by teasing the tissues apart so that the individual eggs were released. The plastic cup was cut into strips and the

Table 1
Number and age of *V. canescens* maintained with no host access

Days	0	1	3	5	7	9	11	13	15	21	27	33	39
Wasps	15	10	13	10	49	11	11	10	13	12	14	14	13

number of eggs deposited on the sides of the cup was counted.

2.4. Statistical analysis

All analyses were performed using JMP v4.0.4 (SAS Institute Inc., 2001).

The three measures of number of eggs matured (deposited, oviduct and total) consistently correlated with wasp age and head capsule width, and so most analyses were performed using a generalised linear model (GLM), with continuous factors centred by their means (Neter et al., 1990). Analyses started with full models with all interactions, and interactions were progressively dropped if $P > 0.25$ (Winer, 1971).

The parameters in the equation predicting total number of matured eggs were determined using a nonlinear regression procedure involving the Gauss–Newton method with step halving. The relation between physical parameters was analysed using the Pearson product–moment correlation coefficient. The relationship between ovigeny index and wasp size was determined using the regression equations of total matured eggs against head capsule width for newly emerged and 1 day-old wasps, and for 39 day-old wasps.

3. Results

3.1. Correlation between physical parameters

Of the three parameters measured, head capsule width returned the highest correlation with dry weight (Table 2) and so was used in subsequent analyses as a measure of wasp size.

3.2. Egg maturation by host-deprived wasps

3.2.1. Lifetime egg maturation by host-deprived wasps

Female *V. canescens* maintained without hosts began to deposit eggs onto the sides of the culture vessel on the day of eclosion. Egg maturation did not cease once the maximum oviduct egg load was reached but rather egg deposition continued for the duration of the experiment (up to 39 days).

Table 2
Correlation between physical parameters of newly emerged *V. canescens*. Sample size is 49

	Wing length	Rear tibia	Head width
Dry weight	0.7839	0.7396	0.8453
Wing length		0.5838	0.8698
Rear tibia			0.6006

Wasps emerged with an average of 39 eggs in their oviducts. In host-deprived wasps, the oviduct egg load increased over the first 3 days and then remained approximately constant (average of 110 eggs) until day 27 before declining to an average of 80 eggs by day 39. On the first day after emergence, wasps deposited an average of 7.5 eggs. Over the first 3 days after emergence, wasps deposited on average a total of 48 eggs, and the cumulative number of deposited eggs then increased over time at a decreasing rate up to a mean of around 480 after 39 days. The mean total eggs matured by 39 day-old wasps was 560 with a highest individual total of 678.

Analysis by GLM for total eggs matured, with age and head capsule width as factors, revealed that total eggs matured varied significantly with age ($F_{1,191} = 1294.4$, $P < 0.0001$) and head capsule width ($F_{1,191} = 98.9$, $P < 0.0001$). There was also a significant interaction between age and head capsule width ($F_{1,191} = 43.0$, $P < 0.0001$). The data for total eggs matured were fitted to an equation of the form:

$$\text{Total eggs matured} = k_1 + (k_2 + k_3 \times \text{head width}) \times (1 - \exp(-k_4 \times \text{age in days}))$$

The total number of matured eggs asymptotically approached a maximum value that was dependent on the size of the wasp, as modelled in Fig. 1. Regression of the predicted total matured eggs against the experimental values returned a value of $r^2 = 95.1\%$ (Fig. 2).

In contrast to the prediction by Jervis et al. (2001), there was a positive relationship between wasp size and ovigeny index (Fig. 3).

3.2.2. Eggs matured by wasps deprived of hosts for 7 days

At 7 days, there was a positive linear relationship between head capsule width and oviduct egg load ($r^2 = 0.438$; $F_{1,46} = 36.7$, $P < 0.0001$), deposited eggs ($r^2 = 0.371$; $F_{1,46} = 27.7$, $P < 0.0001$) and total eggs ($r^2 = 0.847$; $F_{1,46} = 259.8$, $P < 0.0001$) (see Fig. 4a–c). Regression analysis revealed an inverse relationship between the residuals of the trendline plots of oviduct egg load and deposited eggs against head capsule width ($r^2 = 0.568$; $F_{1,46} = 61.7$, $P < 0.0001$) (Fig. 5d). Thus, when corrected for wasp size, the more eggs a wasp had deposited the fewer were present in the oviducts, and vice versa.

3.2.3. Eggs matured by single-oviduct wasps

Over the course of the experiment, seven wasps were found upon dissection to have only one functional

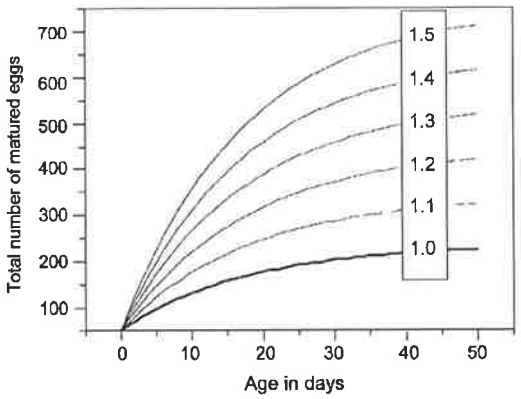

Fig. 1. Predicted number of eggs matured by host-deprived *V. canescens* as a function of age. Insert shows wasp sizes as head capsule width in mm.

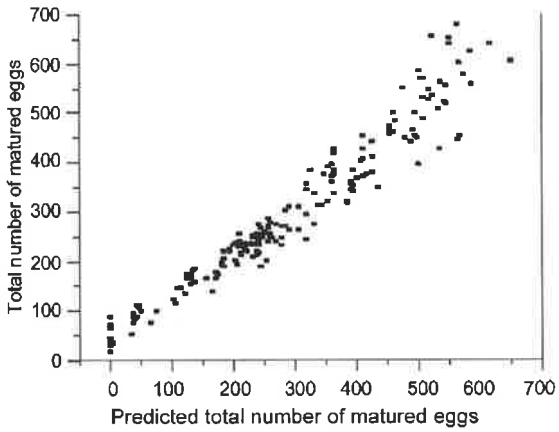

Fig. 2. Predicted vs. observed total numbers of eggs matured by host-deprived *V. canescens* of different ages and sizes.

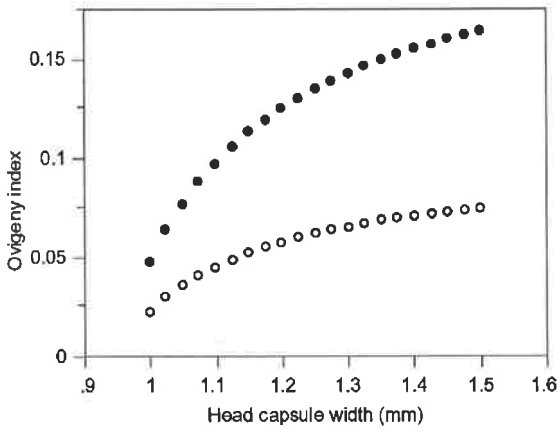

Fig. 3. Relationship between wasp size and ovigeny index. Open circle is the ratio of egg numbers for newly emerged against 39 day-old wasps, closed circle is for 1 day-old against 39 day-old.

ovary, with the second ovary withered and containing no eggs in either the ovarioles or the oviduct. One-oviduct wasps deposited approximately half as many eggs as two-oviduct wasps ($F_{1,193}= 49.3$, $P < 0.0001$; least squares means: one-oviduct wasps = 112.8, two-oviduct wasps = 202.8). However, there was no significant difference in the number of eggs in a single oviduct between one- and two-oviduct wasps ($F_{1,390}= 0.401$, $P= 0.5369$; least squares means: one-oviduct wasps = 51.6, two-oviduct wasps = 47.8).

3.3. The effect of host access on the rate of egg maturation

It is possible that the rate at which *V. canescens* matures eggs is solely determined by metabolic processes, unmodified by any feedback. To test this, 6 day-old wasps were given access to hosts for 24 h and then their rate of egg maturation compared to wasps that had remained host deprived. It was found that wasps given access to hosts matured additional eggs at a higher rate over the following 4 days than those of the same age that were not given access to hosts (Fig. 5).

Analysis by GLM for egg number (total for host access treatment and deposited for the no host access treatment), with day and treatment as factors, revealed that egg number varied significantly with day ($F_{1,37}= 609.4$, $P < 0.0001$) and treatment ($F_{1,37}= 306.5$, $P< 0.0001$). There was also a significant interaction between day and treatment ($F_{1,37}= 64.3$, $P < 0.0001$).

3.4. The effect of crushing the base of the ovipositor on egg maturation

The previous experiment showed that the rate of egg maturation observed in host-deprived wasps does not represent the maximum possible rate. The question remains whether a wasp that is unable to deposit eggs will cease to mature additional eggs once its oviducts are full. To investigate this possibility, the ovipositors of newly emerged wasps were treated with cyano-acrylate glue in order to prevent egg deposition. The wasps were then maintained for 7 days and the total number of eggs matured was determined. All treated wasps continued to deposit eggs although at a reduced rate, indicating at least partial failure of the gluing process. However, while the results were highly variable, total oviduct egg numbers were often much lower than normal, ranging from 24 to 152. These observations suggested that egg maturation might be determined not by oviduct egg load but rather by feedback from the sensilla of the ovipositor. To test this hypothesis, two experiments were conducted.

In the first experiment, newly emerged wasps were chilled on ice and the base of the ovipositor crushed with jewellers' forceps to damage the nerve and hence

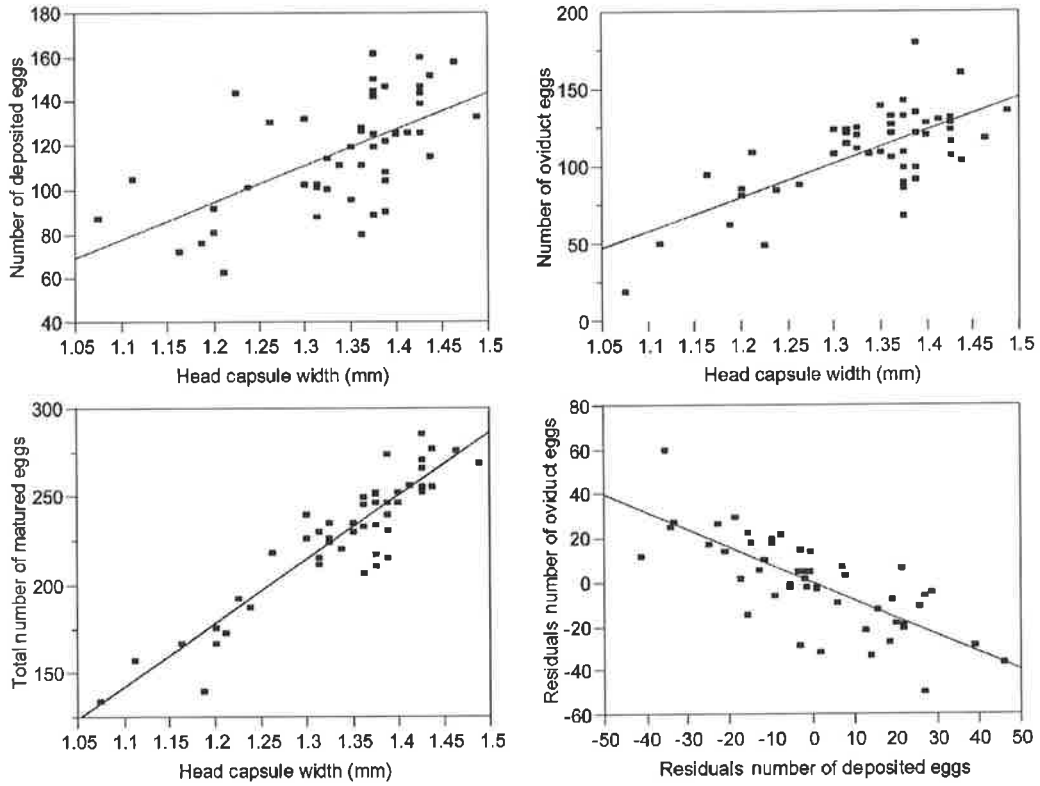

Fig. 4. Relationships between *V. canescens* head capsule width in mm, and number of eggs matured by wasps deprived of hosts for 7 days. (a) Head capsule width vs. number of deposited eggs, (b) head capsule width vs. number of eggs in the oviducts, (c) head capsule width vs. total number of matured eggs, (d) relationship between the residuals of the trendline plots shown in Fig. 3a,b.

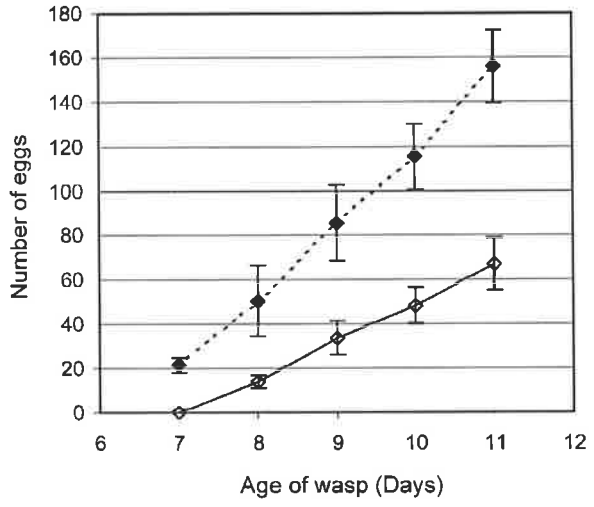

Fig. 5. Mean number of eggs deposited by *V. canescens* that were not given host access and hence were not oviduct depleted (◇), and the mean sum of the eggs deposited and in the oviducts of wasps following 24 h of host access between days 6 and 7 (◆), for wasps at different ages. Bars represent standard errors of the means. Sample size is five at each point.

inhibit any sensory feedback from the ovipositor ("crushed"). As controls, a second group of wasps were chilled on ice and the auxiliary valvulae were cut off at the base without damaging the ovipositor ("faux"). All wasps were maintained for 7 days and total eggs matured determined. The results for the two groups were also compared with those for the 7 day-old wasps described previously, which received no treatment ("untreated").

"Crushed" treatment wasps deposited fewer eggs, and had fewer eggs in their oviducts, compared to "faux" or "untreated" wasps (Table 3). Analysis by GLM showed that deposited eggs ($F_{2,75}= 69.6$, $P < 0.0001$), oviduct egg load ($F_{2,75}= 12.42$, $P< 0.0001$) and total matured eggs ($F_{2,75}= 161.6$, $P < 0.0001$) all varied significantly with treatment.

In the second experiment, 6 day-old wasps were subjected to the treatments "crushed", "faux" and "untreated" as described above. All wasps were then given access to hosts for 24 h. A randomly chosen sample of wasps was dissected immediately after host access and it was found that they were egg depleted, with no significant differences in oviduct egg load between the three groups ($F_{2,22}= 0.0251$, $P= 0.9752$; overall mean was 21.4 eggs). The remaining wasps were

Table 3
Least squares means (LSM) and standard error (SE) of the mean of deposited, oviduct and total eggs for *V. canescens* maintained with no host access for 7 days following treatment of either the base of the ovipositor being crushed (crushed), the auxiliary valvulae being cut off (faux) or no treatment (untreated). Within a column, treatments with the same letter were not significantly different by Tukey's HSD

Treatment	*N*	Deposited eggs		Oviduct eggs		Total eggs	
		LSM	SE	LSM	SE	LSM	SE
Crushed	17	55.4a	4.77	80.1a	5.25	135.8a	4.58
Faux	14	122.7b	5.24	102.5b	5.77	225.1b	5.04
Untreated	49	117.6b	2.80	110.5b	3.09	229.5b	2.69

maintained for a further 4 days and then the total number of eggs matured was determined.

As in the first experiment, "crushed" treatment wasps deposited fewer eggs, and had fewer eggs in their oviducts, compared to "faux" or "untreated" wasps (Table 4). Analysis by GLM showed that deposited eggs ($F_{2,24}= 16.0$, $P < 0.0001$), oviduct egg load ($F_{2,24}= 10.3$, $P= 0.0004$) and total matured eggs ($F_{2,24}= 21.7$, $P < 0.0001$) all varied significantly with treatment.

4. Discussion

Previously, it was thought that *V. canescens'* rate of egg deposition (into hosts) depended on the rate of searching behaviour, which was related to the oviduct egg load. Egg maturation continued until the carrying capacity of the oviducts was reached, at which point oogenesis was inhibited. If a wasp subsequently encountered hosts, then the oviduct egg load would fall below the maximum and oogenesis would restart. However, in this study of *V. canescens* maintained without hosts, egg maturation did not cease once the maximum oviduct egg load was reached but rather continued for the duration of the experiment, with increasing numbers of eggs being deposited onto the sides of the culture vessel.

These data raise two connected questions. The first issue is, why does *V. canescens* deposit eggs in the absence of hosts? Noting that single-oviduct wasps deposited around half the number of eggs as two-oviduct wasps, but were not egg depleted, suggests that the phenomenon does not have a strictly behavioural basis, but rather is due to properties of the individual oviduct, relating to the transfer of eggs toward the ovipositor. Since newly emerged wasps, and wasps that had had their ovipositors crushed both deposited eggs at a time when their oviducts were not full further indicates that the effect is not exclusively due to the need to deposit additionally matured eggs once the oviducts are full. However, the movement of an egg from the oviduct to the tip of the ovipositor is not simply a continuation of the movement down the oviduct but requires a lateral flexing of the ovipositor (Rogers, 1972). Thus, the implication is that the involuntary movement of eggs down the oviduct, aided by the ongoing maturation of additional eggs, presents eggs at the base of the ovipositor from where they are transferred to the tip and, in the absence of hosts, eventually deposited, either by accident during host seeking, by necessity due to the upstream pressure of additionally matured eggs, or by design as they degenerate in the oxidising environment. Under this model, egg load is only indirectly related to the rate of host seeking behaviour, by increasing the rate at which eggs are presented for transfer to the tip of the ovipositor.

Whether the phenomenon of egg deposition under conditions of host deprivation is restricted to *V. canescens* is not known. However, one of its consequences, oviduct egg depletion in the absence of hosts, has been reported in a number of studies (e.g. Collier, 1995; Tran and Takasu, 2000). Egg depletion is often inter-

Table 4
Least squares means (LSM) and standard error (SE) of the mean of deposited, oviduct and total eggs for *V. canescens* given host access for 24 h between days 6 and 7 and then maintained for a further 4 days without hosts, following treatment of either the base of the ovipositor being crushed (crushed), the auxiliary valvulae being cut off (faux) or no treatment (untreated). Within a column, treatments with the same letter were not significantly different by Tukey's HSD

Treatment	*N*	Deposited eggs		Oviduct eggs		Total eggs	
		LSM	SE	LSM	SE	LSM	SE
Crushed	10	6.30a	2.78	77.8a	8.16	84.1a	7.95
Faux	10	28.6b	2.86	124.8b	8.4	153.5b	8.18
Untreated	9	22.4b	3.77	127.2b	11.1	149.6b	10.79

preted as evidence for oosorption; the results of this study indicate this is not always the case.

The second issue raised by the data is, what determines the rate of oogenesis, in terms of both metabolism and control? In the present study, it was found that egg maturation by host-deprived wasps continued after the oviducts were full. This raises the possibility that the rate at which *V. canescens* matures eggs is solely determined by metabolic processes, unmodified by any feedback. However, following host access and oviduct depletion, the rate at which additional eggs were matured increased. Clearly, the rate of oogenesis can be elevated by feedback. When the ovipositor of newly emerged wasps was crushed at the base, both egg deposition and oviduct egg load were reduced. This indicates that the rate at which untreated wasps matured eggs is not some metabolically uncontrollable minimum, and further that egg maturation is not stimulated solely by the oviducts being below their carrying capacity. By inference, this suggests that the rate of egg maturation is modified by sensory feedback from the ovipositor. Consistent with this explanation, wasps given access to hosts after their ovipositors were crushed depleted their oviducts to the same extent as untreated wasps but subsequently matured fewer eggs. Since in all cases, following treatment, at least some additional eggs were produced, however, it is possible that there is a minimum rate of egg maturation, or that the rate is also controlled in part by feedback on oviduct egg load.

The above interpretation assumes that the process of damaging the ovipositor did not itself significantly affect the metabolic activity of the wasp. This is reasonable, since the results for wasps that were treated by having their auxiliary valvulae cut off were not significantly different from untreated controls; however, the possibility cannot be excluded, either theoretically or experimentally.

At the metabolic level, the rate-determining factor of oogenesis must, in broad terms, involve either the separation of follicles from the germ cells, the synthesis of precursor molecules by the fat body or their utilisation by the follicles. In this study, it was found that the total number of eggs matured by a host-deprived wasp could be fitted to an equation of the general form:

$$\text{Total eggs matured} = k_1 + (k_2 + k_3 \times \text{head width}) \times (1 - \exp(-k_4 \times \text{age in days}))$$

It can be shown from this equation that, at any time, the daily rate at which eggs are matured is a constant fraction of the number of potential eggs remaining to be matured, with the value $(e^{k4}) - 1$. Assuming the number of eggs *V. canescens* can mature is directly proportional to its fat reserves, this speculatively suggests that, under the constant environmental condition of host deprivation and potentially at other times as well, the rate of oogenesis is controlled by controlling the rate of synthesis of precursor molecules by the fat body. This hypothesis will be explored in future research.

Whatever the underlying causes are, the consistency of the rate at which individual females mature eggs, and the inverse relationship observed between the numbers of deposited and oviduct eggs indicates that even under conditions of host deprivation, *V. canescens* is effectively unable to completely inhibit oogenesis. Interestingly, while it has been shown that oosorption by anhydropic egg producers can occur under conditions of nutrient stress (reviewed in Bell and Bohm, 1975), in a number of species of encyrtid wasps under conditions of host deprivation, oogenesis and oosorption occur simultaneously (Flanders, 1942; Rivero-Lynch and Godfray, 1997). The risk of precocious development can be ruled out as a cause as oviposition must occur for embryogenesis to begin (Flanders, 1942). One possibility is that encyrtid eggs, once mature, quickly decline in quality (Rivero-Lynch and Godfray, 1997). Alternatively, it is possible that, like *V. canescens*, encyrtids are unable to completely inhibit oogenesis. The hydropic eggs of *V. canescens* contain little salvageable material and so surplus eggs are discarded; in contrast, the anhydropic eggs of the encyrtids are nutrient-rich and so are resorbed.

In summary, the study found that *V. canescens* females maintained without hosts deposited eggs onto the sides of the culture vessel. Egg maturation did not cease once the maximum oviduct egg load was reached but rather continued for the duration of the experiment (up to 39 days), at a rate of around 5.8% of the remaining unmatured eggs per day. The rate of egg maturation increased following host access but decreased following artificial damage to the ovipositor, even though the oviducts were partly egg depleted. The results suggest that the rate at which eggs are matured may be determined by the rate of synthesis of precursors by the fat body, which in turn is modified by feedback from the ovipositor. Further, they suggest that the discarding of eggs is due to the involuntary unidirectional movement of eggs down the oviduct, facilitated by the ongoing maturation of additional eggs.

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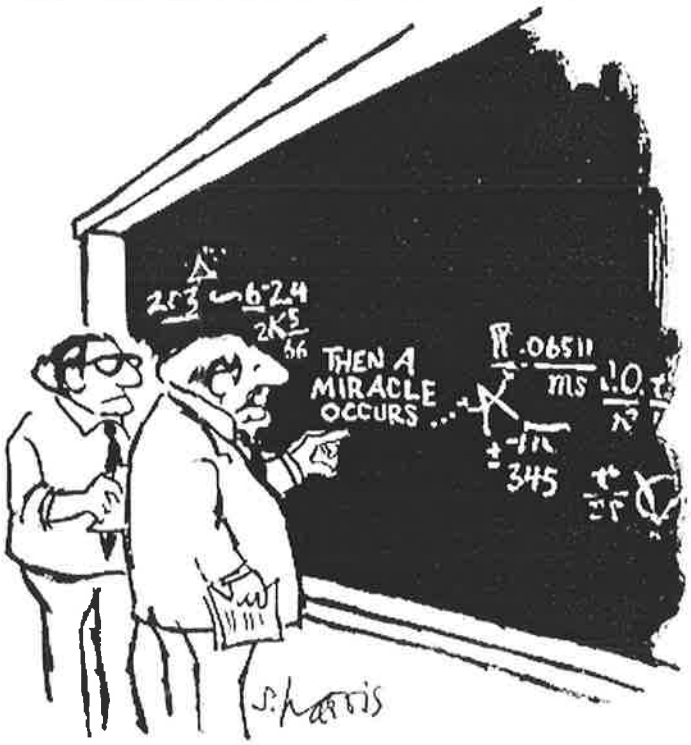
Chapter Seven

An empirical model of the sympatric coexistence of two strains of the endoparasitoid wasp *Venturia canescens*



"I think you should be more explicit here in step two."

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Harry L.S. Roberts (Candidate)
Designed experiments, performed experimental work, analysed and interpreted data, formulated model, wrote manuscript and acted as communicating author.

Michael Keller (Co-supervisor) Assisted with formulation of model

Otto Schmidt (Principal Supervisor)
Supervised work and helped in manuscript preparation.

Signed (Harry Roberts)

.....*Date*.....

Signed (Otto Schmidt)

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An empirical model of the sympatric coexistence of two strains of the endoparasitoid wasp *Venturia canescens.*

Harry L.S. Roberts*, Michael Keller and Otto Schmidt

Insect Molecular Biology Laboratory, Department of Plant and Pest Science, The University of Adelaide, Glen Osmond SA 5064, Australia

* corresponding author
email: harry.roberts@adelaide.edu.au
phone: +61-83037274
fax: +61-83794095
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Abstract

Recent research has demonstrated that a laboratory culture of the asexual solitary endoparasitoid wasp *Venturia canescens* Grav. (Hymenoptera: Ichneumonidae) contains two genetically and phenotypically distinct lines, coexisting on their host the flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). The basis to the coexistence of the two lines appears to be differences in their reproductive success under single parasitism and superparasitism. Further, examination of field-derived wasps from several locations has shown that the phenotypes displayed by the laboratory colonies also co-occur in field populations.

Historically, the impossibility of showing that two species do not occupy separate niches has precluded any demonstration of sympatric coexistence in the field. Here we present the results of an iterative model that uses a range of experimental life history data to predict the stable composition of a mixed population of two lines displaying the laboratory phenotypes under different rates of superparasitism. The model predicts that sympatric coexistence of the two lines is possible when the overall rate of superparasitism is between 4 and 12% or greater. These values are within the rates reported for other solitary endoparasitoid wasp species in the field, and so demonstrate that the sympatric coexistence under natural conditions of two species that display the phenotypes observed in the laboratory lines is, in principle, possible.

Introduction

The principle of competitive exclusion (Volterra, 1926; Gause, 1934) states that it is impossible for two species that are limited by the same resource to coexist indefinitely. While mathematical models incorporating non-linear phenomena suggest that sympatric coexistence is possible under certain conditions (e.g. Levins, 1979; Armstrong and McGehee, 1980; Durret and Levin, 1998), the basic theory remains popular (Vandermeer et al., 2002). In part, this is due to the numerous examples in the literature of competitive displacement of one species by another, but also because of the difficulty of demonstrating that there is no niche differentiation involved in those cases where species have been shown to be coexisting (see DeBach, 1966). However, recent research has demonstrated that a laboratory culture of the asexual (=thelytokous) parasitoid wasp *Venturia canescens* contains two genetically distinct lines, coexisting on their host the flour moth *Ephestia kuehniella* (Hellers et al., 1996; Beck et al., 1999, 2000, 2001).

The two lines are addressed as RP (repeat plus) and RM (repeat minus) for the presence or absence of a 54 base-pair tandem repeat sequence in the gene coding for a virus-like particle (VLP1) protein (Hellers et al., 1996). The lines are genetically stable, and differ in a range of phenotypic characters, including ovarian morphology, calyx gland secretions and reproductive success (Beck et al., 1999, 2000, 2001). Since the laboratory culture had been maintained without the addition of new stock for over 400 generations, the two lines must have been coexisting sympatrically while competing for the same resource.

V. canescens lays eggs directly into the body of the larva of its host (=endoparasitic), where the developing parasitoid feeds on the haemolyph. When more than one egg is deposited into a host, it is described as superparasitised. However, irrespective of the number of eggs laid, at most a single wasp emerges from a host (= solitary). When the time interval between ovipositions is around three days or less, parasitoids fight for possession of the host using strong sickle-shaped mandibles to attack competitors, and the outcome is uncertain. For greater time intervals the older larvae prevails, probably by suppressing the development of its younger adversary by anoxia (Fisher, 1961, 1963)

The basis to the coexistence of the two lines appears to be differences in their reproductive success under single parasitism and superparasitism. In a simulation of laboratory conditions, Beck et al. (1999) observed that when RM- and RP-wasps were allowed to compete for hosts for a 24-hour interval the RM-wasps produced significantly more offspring than the RP-wasps. However, under intra-line competition the RM-wasps produced significantly fewer offspring than the RP-wasps. Reineke et al. (2004) examined the reproductive success of the two lines when each competing female was allowed to oviposit only a single egg into each host, with the second female ovipositing after a lag time of four to 48 h. For the RM-line the relative pay-off of superparasitism was around 65% when the time interval between ovipositions was a few hours, declining to 50% when the time interval was 2 days. In contrast, the relative pay-off for the RP-line was around 40% independent of the time interval between ovipositions. Dissections of supernumery larvae from superparasitised hosts indicated that about a third of contests were resolved around the time the younger larva hatched, and in almost all cases within 24 hours, corresponding to the period when physical combat is the dominant mode of competition (Fisher, 1961).

Investigations of interlarval combat under *in vitro* conditions (Roberts et al., 2004b) showed that the outcome of interlarval combat depended on both the relative and absolute ages of the two competing larvae. The higher reproductive success of the RM line under conspecific superparasitism (Reineke et al., 2004) appears to be due to a physiological difference between the newly hatched larvae of the two lines, which results in an advantage to the RM larva irrespective of the order or time interval between ovipositions.

The research described above demonstrates that the two lines have been coexisting for many generations under laboratory conditions, that they differ in key life history traits that impact on fitness, and suggests a mechanism for their sympatric coexistence. Further, since the lines are asexual, they are reproductively isolated from each other, and hence effectively behave as separate species. However, the question remains whether such a system can be stable under natural conditions, or whether it is only possible in the laboratory-created environment.

An examination of field-derived asexual wasps from Mildura, Australia and Mt. Boron, Southern France, using the VLP1 gene as a marker, found that wasps displaying phenotypic clusters qualitatively similar to the laboratory RM- and RP-lines co-occur in natural populations (Li et al., 2003). Homozygous RM and RP offspring derived from a mixed culture of field-collected sexual (=arrhenotokous) wasps also showed similar patterns of morphological and functional differences to the laboratory lines. In all three populations, the phenotype observed corresponded to the VLP1 allele but the quantitative expression of the phenotype was less pronounced, suggesting the VLP1 gene locus is genetically linked to the phenotype and that a second unlinked gene is also involved (Li et al., 2003). In a second study, Amat et al. (2003) reported asexual RM and RP females derived from material collected in Antibes both displayed a laboratory RM-like phenotype, confirming that the phenotypes displayed by the laboratory colonies also occur in field populations but suggesting the correlation observed by Li et al. (2003) may be coincidental. Alternatively, it is possible that the laboratory RM-phenotype is the result of more than one gene, acting additively but able to act separately. Given the relative competitive advantage of the RM-phenotype under conditions that favour superparasitism (Beck et al., 1999; Reineke et al., 2004), it would not be surprising if strains displaying different pathways to the RM-phenotype occurred in field populations.

The fact that wasps displaying RM- and RP-phenotypes have been found in the same geographical locations does not demonstrate that they are coexisting sympatrically. No amount of field data will be able to exclude the possibility that the two phenotypes occupy separate niches. However, it is possible to estimate by mathematical modelling the conditions under which two sub-populations displaying the same life history parameters as the two laboratory lines can coexist in competition for the same host resource. In this study those parameters that are not in the literature were first determined. Then, the experimentally derived data set was applied to an iterative model to predict the stable composition of RM and RP wasps in a mixed population under different rates of superparasitism.

Material and Methods

Rearing procedures

Wasps were clonal RP- and RM-wasp lines from a thelytokous *V. canescens* laboratory culture as reported by Beck et al. (1999). For general colony maintenance, wasps were reared in cylindrical clear plastic tubs (height 20cm and diameter 15cm). Four or five adult wasps were placed into each container with 40-50 hosts until the wasps died (around 36 h). Upon emergence the wasps were removed from culture and kept in gauze-covered 425 ml clear plastic cups (Party Rite Jumbo Tumblers™, Harris Paper Pty. Ltd., West Heidelberg, Australia) with a 50% honey solution. Hosts were *E. kuehniella,* reared on a 10:2:1 mixture of oat bran, wheat germ, and dried brewers yeast. All experiments were conducted and cultures maintained at 25 ± 1°C, under a constant light-dark regime (L14:D10).

Parasitism procedures

To obtain singly parasitised *E. kuehniella,* a single wasp was put together with 25-30 host larvae of varying sizes in a plastic container (7 cm in diameter, 8 cm high). The parasitoids were observed during oviposition and stinging attempts that resulted in a startle response from the larvae, combined with the characteristic cocking movements of the wasp's ovipositor (Rogers, 1972) were considered as real oviposition events. Any stinging attempt that either did not evoke a startle response or was not followed by a cocking movement was regarded as uncertain and the larva was discarded. After parasitisation the hosts were weighed and maintained individually in glass vials with excess food until parasitoid eclosion. Superparasitised *E. kuehniella* were obtained similarly, by presenting wasps with hosts that had already been parasitised once.

Measurement of phenotypic characters

Emergent wasps were killed by freezing and the time and date of emergence recorded, which allowed development time to be calculated following molecular genotyping to determine the line of the wasp. Head capsule width was measured to the nearest 0.0125 mm with an optical micrometer. Molecular genetic analyses were performed as per Reineke et al. (2004).

Total eggs matured by 7-day-old host-deprived wasps were determined as per Roberts and Schmidt (2004).

Statistical analysis

Data were analysed using the generalised linear model (GLM) platform, JMP V4.0.4 (SAS Institute Inc., 2001), with continuous factors centred by their means (Neter et al., 1990). Analyses started with full models with all interactions, and interactions were progressively dropped if P>0.25 (Winer, 1971).

An empirical model of the sympatric coexistence of the two strains

The model uses an iterative approach with experimental data to predict the stable composition of RM and RP wasps in a mixed population under different rates of superparasitism. The major simplifying assumptions of the model are that the generations do not overlap, and that no host is parasitised more than twice.

The fraction of superparasitised hosts is defined as some number A, and the fraction of single parasitised hosts is $(1-A)$. Each generation the fraction of adult wasps for each strain will be:

Adult wasps = (relative offspring from superparasitism) $* A$ + (relative offspring from parasitism) $* (1-A)$

In general, the reproductive output (RO) of each strain will be the product of the fraction of hosts parasitised (HP) and the survival rate of wasps from parasitised hosts (SR), such that:

$$RO = HP * SR$$

In general, the fraction of hosts parasitised will be the product of the relative rate of encounter (ER) and the rate of acceptance (AR), such that:

$$RO = ER * AR * SR$$

The reproductive value (RV) of those wasps will also depend on the effects on fecundity of differences in development time and adult size, represented by a fecundity modifier term (FM), such that:

$$RV = RO * FM = ER * AR * SR * FM$$

For the i^{th} generation,

the fraction of RM females = M_i

the fraction of RP females = P_i

Under single parasitism, the relative encounter rate will be the fraction of each strain in the population (M_i and P_i), and the rate of acceptance is assumed to be the same for each strain and so is set to one. The rate of single parasitism is defined as (1-A), and so, with the subscripts m and p referring to the RM and RP strains respectively, the reproductive value from single parasitism for the each strain is:

$RVsingle(RM)_i = M_i * SR_m * FM_m * (1-A)$

$RVsingle(RP)_i = P_i * SR_p * FM_p * (1-A)$

Under superparasitism, the relative encounter rate will be the products of the relevant relative fraction of each strain in the population, such that:

ER(RM first, RM second) = M_i^2

ER(RP first, RP second) = P_i^2

ER(RM first, RP second) = $M_i * P_i$

ER(RM first, RM second) = $P_i * M_i$

The acceptance rate will be the relative probability of a female superparasitising a host containing either an egg of the same (AR_s) or a different (AR_d) strain, and so the relative fraction of hosts superparasitised under each of the four combinations will be:

$HP_{mm} = M_i^2 * AR_s$

$HP_{pp} = P_i^2 * AR_s$

$HP_{mp} = M_i * P_i * AR_d$

$HP_{pm} = P_i * M_i * AR_d$

These values are then normalised to have a sum of one, for example:

$HP\hat{}_{mm} = M_i^2 * AR_s / (M_i^2 * AR_s + P_i^2 * AR_s + M_i*P_i * AR_d + P_i*M_i * AR_d)$

The survival rate of wasps (SR) of each strain from superparasitised hosts can vary with the order of oviposition. The subscript xy refers to the first and second ovipositing female respectively and the strain of the emergent wasp is indicated by capitalisation, for example:

RM adults from hosts parasitised by RM then RP = SR_{Mp}

RP adults from hosts parasitised by RM then RP = SR_{mP}

Thus, the relative reproductive output of each strain under superparasitism will be:

$RO_m = HP\hat{}_{mm} * SR_{mm} + HP\hat{}_{mp} * SR_{Mp} + HP\hat{}_{pm} * SR_{pM}$

$RO_p = HP\hat{}_{pp} * SR_{pp} + HP\hat{}_{mp} * SR_{mP} + HP\hat{}_{pm} * SR_{Pm}$

For a given rate of superparasitism, defined as A, the reproductive value of adults from superparasitised hosts from each strain is:

$RVsuper(RM)_i = RO_m * FM_m * A$

$RVsuper(RP)_i = RO_p * FM_p * A$

And the overall relative fraction of each strain in the $(i+1)^{th}$ generation, from single and superparasitism combined, is:

$M_{i+1}(relative) = RVsuper(rm)_i + RVsingle(rm)_i$

$P_{i+1}(relative) = RVsuper(rp)_i + RVsingle(rp)_i$

These fractions are then normalised to have a sum of sum one, giving the fraction of each strain in the $(i+1)^{th}$ generation:

$M_{i+1} = M_{i+1}(relative) / [M_{i+1}(relative) + P_{i+1}(relative)]$

$P_{i+1} = P_{i+1}(relative) / [P_{i+1}(relative) + M_{i+1}(relative)]$

For each given set of parameters the model is then run iteratively for different values of *A* (the fraction of superparasitised hosts) until there is no change in the value for the fractions of the two wasp lines.

The model requires the experimental estimation of three parameters, the rates of emergence of wasps from hosts (*SR*), a fecundity modifier (*FM*) term to incorporate developmental differences, and the rates of acceptance of encountered hosts (*AR*).

Survival rates (*SR*) under single parasitism are calculated from the present study, under intra-line superparasitism from Beck et al. (1999) (Table 1) and from inter-line superparasitism from Reineke et al. (2004) (Table 2).

Fecundity modifier (*FM*) values (Table 3) are based on the relations between host mass and adult size (Figs. 1a, b), and the relation between wasp size and lifetime egg maturation described in Roberts and Schmidt (2004), with the value for the RP strain under single parasitism treated as the baseline. Parameters are calculated as least squares means for hosts ranging from around 10 to 50mg, corresponding to late second through to final instars (Harvey, 1996). While lifetime egg maturation of the RM-line was not determined, there was no significant difference in the relation between adult size and the number of eggs matured by 7-day-old females of the two lines (Fig. 2) and so the use of the RP-line relation for both is reasonable. The effects of parasitoid line and parasitism status on development time were not significant.

The values for rates of acceptance under superparasitising conditions are derived from a laboratory study by Marris et al. (1996), who reported that for *V. canescens* parasitising the Indian meal moth *Plodia interpunctella* host acceptance rates for wasps presented with a host containing their own egg, a sibling's egg and an egg of a different strain were 0.2282, 0.4534 and 0.7031 respectively. The relative rate of acceptance (*RA*) of a host by a superparasitising female is calculated as the ratios of those same-strain rates to that for a host containing an egg of a different strain (Table 4).

Results

Survival of hosts containing a single egg

Analysis of the rate of emergent adults from singly parasitised hosts with strain and host mass as factors revealed the rate of adults from hosts was significantly lower (X^2 = 7.368, df = 2,197, p = 0.0066) for the RM strain compared to the RP strain (Table 1), however the effect of host mass was not significant. An analysis of the stage at which developmental failure occurred revealed that compared to RP-line significantly more (X^2=4.837, df=1,51, P=0.0278) RM parasitoids failed as late instars (Fig. 3). Failed larva generally were not encapsulated or melanised.

Development time and adult mass

Analysis of development time by GLM with parasitism status, strain of emergent wasp and host mass as factors, revealed a small but significant positive relationship between host mass and development time (F=8.834, df=1,282, P=0.0032, Rsquare=0.032), while the effects of both strain and parasitism status were not significant (Table 5). A similar analysis on wasp head capsule width revealed significant effects for parasitism status (F=186.0, df=1,270, P<0.0001), strain (F=43.74, df=1,270, P<0.0001) and host mass (F=205.8, df=1,270, P<0.0001) as well as a significant interaction (F=11.5, df=1,270, P=0.0008) between parasitism status and host mass (Figs. 1a, b).

Egg maturation

Analysis of total eggs matured by 7-day-old host-deprived wasps with strain and wasp size as factors revealed that the number of matured eggs increased

with wasp size ($F=477.2$, $df=1,82$, $P<0.0001$), while there was no significant difference between the strains (Fig. 2).

The model

Two of the parameters relating to superparasitism that are required in the model can take a range of possible values. The host acceptance rate for intra-line superparasitism depends on whether the two eggs are laid by the same, or by different wasps. When the model is run for $A=0$ (no superparasitism) irrespective of the initial frequencies of the two lines the RP-line always drives the RM-line to extinction. The model seeks to estimate the lowest rate of superparasitism under which the two lines will coexist indefinitely. As this will correspond to the situation where the RM-line is present as a very small fraction of the total population, the fraction of intra-RM-line superparasitism involving two different RM-females will be negligible, and can be ignored. In contrast, the RP-line will comprise the greater fraction of the population, and so intra-RP-line superparasitism will involve both self-superparasitism and eggs being laid by two different RP-females.

In addition, the survival rates for the two lines under inter-line superparasitism vary with the time interval between ovipositions. While in theory superparasitism could occur over any time interval up to the emergence of the wasp (around 24 days), research by Hubbard et al. (1987) suggests that in the field superparasitism is uncommon when the time interval between ovipositions is greater than 24 hours. Further, it is likely that for a considerable amount of time over a 24 hour period a host is in a refuge or otherwise not accessible by a parasitoid, so that the distribution of the time interval between ovipositions under natural conditions will show a bias towards time intervals at the shorter end of the scale.

To estimate the distribution, under natural conditions, of the time interval between ovipositions or the relative frequencies of intra-RP-line superparasitism that will involve the same or two different wasps, is beyond the scope of the model. Instead the approach taken is to take the extremes of these parameters and regard them as the boundary conditions. The lower boundary for the minimum rate of superparasitism under which the two lines will coexist indefinitely is the condition where two wasps search the same patch at the same time. The survival rates are for a time interval between

ovipositions of between zero to four hours and the intra-RP-line acceptance rate is for self-superparasitism. The parameters that result in the upper boundary correspond to the survival rates being for a time interval between ovipositions of between zero to four hours and the intra-RP-line acceptance rate is for superparasitism by two different wasps. It must be stressed that neither of these scenarios are regarded as realistic; their purpose is to define the limits for the minimum rate of superparasitism under which sympatric coexistence of the two lines will occur.

For a given rate of superparasitism the stable ratio of the two lines is independent of the initial ratios. The results of the model indicate that the minimum rate of superparasitism under which the two lines will coexist indefinitely is between 4 and 12% (Fig. 4a). Above these values the fraction of the RM-line then rises sharply as the rate of superparasitism increases.

The model can also be applied to investigate the stable ratios of the two lines under high levels of superparasitism, reflecting laboratory colony culture conditions. However, as the level of superparasitism rises, the assumption that a single host contains no more than two eggs becomes increasingly invalid and while not worthless the results must be viewed with caution. In this situation, the boundary conditions are defined as above, except that the host acceptance rates for the two lines are reversed. Under the model, at very high levels of superparasitism the two lines continue to coexist indefinitely, with the RM-line comprising a maximum fraction of between 69 and 83% of the population, when the rate of superparasitism is 100% (Fig. 4b).

Discussion

Life history data

The rate of emergent adults from hosts containing a single egg was significantly lower for the RM-line compared to the RP-line (Table 1). This is consistent with the observation by Beck et al. (1999), that when RM- and RP-females engage in intra-line competition for 24 hours the RM-wasps produced significantly fewer offspring than the RP-wasps. The lower rate of parasitism success for the RM-line appears to be due to an increase in the rate of failure as late instars (Fig. 3). There was no evidence that larvae had succumbed to the host's immune response, and so the cause of failure is unclear.

Wasps emerging from hosts containing two eggs were larger than wasps from equivalent sized hosts containing one egg. This is consistent with the results of Roberts et al. (2004a), and may be related to a putative concentration dependent capacity of maternal secretions to modify host physiology. Adult RM-wasps were larger than RP-wasps from equivalent sized hosts under both single egg and superparasitism (Figs. 1a, b). This is consistent with the observation that 8-day-old RM-larvae are larger than corresponding RP-larvae (Reineke et al., 2004), but the basis is unknown.

The Model

Previous research has shown that, under laboratory culture conditions, two lines of the asexual solitary endoparasitic wasp *V. canescens,* differing in a cluster of phenotypic characters, coexist sympatrically on their host the flour moth *E. kuehniella* (Hellers et al., 1996; Beck *et al.* 1999, 2000, 2001). Laboratory experiments have indicated the coexistence of the two lines is due to reciprocal differences in their reproductive success under single egg and superparasitism (Reineke et al., 2004a), with the higher reproductive success of the RM-line under conspecific superparasitism being due to an advantage of newly-hatched RM-larvae in the interlarval physical combat that determines possession of the host (Roberts et al., 2004b). Further, investigations of natural populations from several locations has revealed that wasps displaying similar clusters of phenotypic traits to both laboratory lines occur in the field and can be collected from the same locations (Li et al., 2003; Amat et al., 2003).

A simulation of laboratory colony culture conditions (Fig. 4b) returned the result that the two lines will continue to coexist indefinitely, with the RM-line never exceeding 83% of the population (Fig. 4b). Applications of the model to situations with high levels of superparasitism can only be regarded as suggestive, since the assumption that a single host contains no more than two eggs becomes increasingly invalid as the level of superparasitism rises. However, the results support the suggestion that it is differences in the reproductive success of the two lines under single egg and superparasitism that underlies their observed continued coexistence.

Application of the model to determine the minimum rate of superparasitism required for the stable coexistence of a mixed population of two lines

displaying the laboratory RM- and RP-phenotypes, predicts that sympatric coexistence is possible when the overall rate of superparasitism is between 4 and 12% or greater (Fig. 4a). While no studies have reported on the incidence of superparasitism by *V. canescens* in the field, three lines of evidence suggest that the rate under natural conditions may be above the threshold required to allow coexistence.

Firstly, appropriate superparasitism rates been have reported from field studies involving other species of solitary endoparasitic hymenoptera. Examples include 19.1% for *Collyria calcitrator* on *Cephus pygmaeus* (Salt, 1931, 1932)), 20% for *Limnerium validum* on *Malocosoma Americana* and 7.8% for *Ibalia leucospoides* on *Sirex cyaneus* (Salt, 1934), 10% for *Asobara tabida* on *Drosophila melanogaster* (Janssen, 1989) and 34% for *Pauesia sp.* on *Cinara cronartii* (Kirsten and Kfir, 1991).

Secondly, work by Hubbard et al., (1987) and Marris et al., (1996) on superparasitism avoidance shows that *V. canescens* has the ability to make fine distinctions, between non-parasitised hosts and hosts containing an egg laid by herself, a sibling or a conspecific, with the acceptance rates for the various parasitised hosts ranging from 20 to 70%. This indicates that superparasitism avoidance is only partial and parasitised hosts are likely to be superparasitised if encountered. Indeed, in laboratory trials where single wasps were allowed access to patches containing 20 hosts for one hour, 18% of parasitised hosts were superparasitised (Roberts, unpublished data).

Thirdly, egg maturation by *V. canescens* does not cease once the oviduct storage capacity is reached but is continuous for the life of the wasp. Further, a female without access to hosts dumps eggs, a phenomenon that occurs irrespective of whether the oviducts are at capacity (Roberts and Schmidt, 2004). Although the rate of egg dumping varies with the age, size and previous host access history of each wasp, it is clear that periodically *V. canescens* must accept an already parasitised host or discard an egg with no chance of a payoff.

While they are plausible, in the absence of field data on superparasitism rates it is impossible to conclude whether the rates required by the model occur in the field. In addition, it is always possible that the two phenotypes occupy overlapping but not identical niches. Thus, the results of the model do not and

cannot provide proof that natural populations of *V. canescens* contain two sympatrically coexisting lines. However, since the two lines of *V. canescens* are asexual and so in a reproductive sense separate species, the model does demonstrate that, under ecologically realistic conditions, the sympatric coexistence of two solitary endoparasitoid species that display the phenotypes observed in the laboratory lines is, in principle, possible.

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Tables

Table 1. Mean rate of adult emergence of *V. canescens* from hosts under single parasitism (present study), and intra-line superparasitism (Beck 2000).

Line	RM	RP
Single parasitism	0.75	0.88
Superparasitism	0.64	0.80

Table 2. Mean rate of adult emergence of *V. canescens* from hosts under inter-line superparasitism for different ranges of times between ovipositions (Reineke et al 2004).

Time between ovipositions (h)	RM oviposits first		RP oviposits first	
	RM wins	RP wins	RM wins	RP wins
0 to 4	0.38	0.28	0.51	0.25
0 to 24	0.35	0.25	0.4	0.25

Table 3. Least squares means for the relationship between host mass and head capsule width of *V. canescens*, and the corresponding lifetime potential fecundity (Roberts 2004).

Line	Single parasitism			Superparasitism		
	Head width (mm)	Potential fecundity	Relative fecundity	Head width (mm)	Potential fecundity	Relative fecundity
RM	1.281	478	1.044	1.374	563	1.229
RP	1.261	458	1	1.337	528	1.153

Table 4. Host acceptance rates under intra- and inter-strain superparasitism by *V. canescens* (Marris et al 1997).

Superparasitising Line	Self vs conspecific		Sibling vs conspecific	
	Acceptance rate	Proportion acceptance	Acceptance rate	Proportion acceptance
Same	0.23	0.25	0.45	0.39
Conspecific	0.70	0.75	0.70	0.61

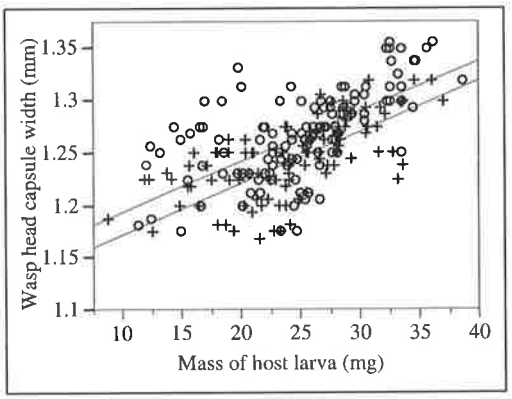
Table 5. Mean development time (days) of *V. canescens* from hosts under single parasitism and inter-line superparasitism.

	Single parasitism		Inter-line superparasitism	
Line	RM	RP	RM	RP
N	44	47	118	102
Mean	23.7	23.8	23.5	23.6
SD	1.0	1.0	1.3	1.0

Figures

Figure 1a. Relationship between the mass of *E. kuehniella* and adult *V. canescens* head capsule width under a) single parasitism and b) inter-line superparasitism. Open circle and solid line represents RM wasps, plus and dotted line represents RP wasps.

1a



1b

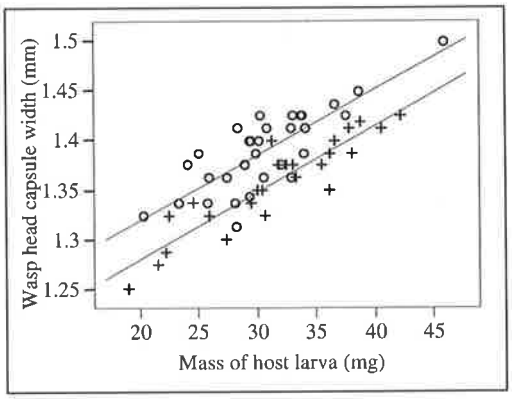


Figure 2. Relationship between total matured eggs and head capsule width for 7-day-old host-deprived *V. canescens*. Open circle and solid line represents RM wasps, plus and dotted line represents RP wasps.

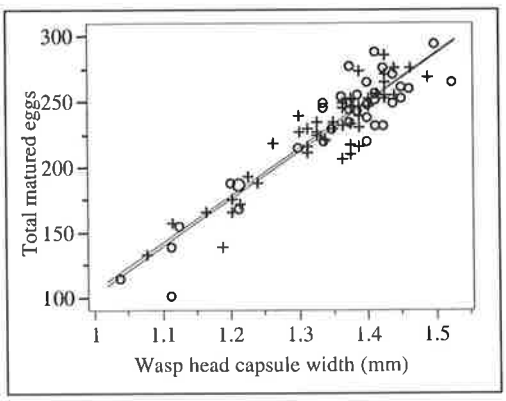


Figure 3. Stage of RM- and RP-line *V. canescens* larvae in single parasitised *E. kuehniella* when developmental failure occurred, as a fraction of total hosts. Black represents early larva, diagonal bars represents late larva/pupa and grey represents pharate adult.

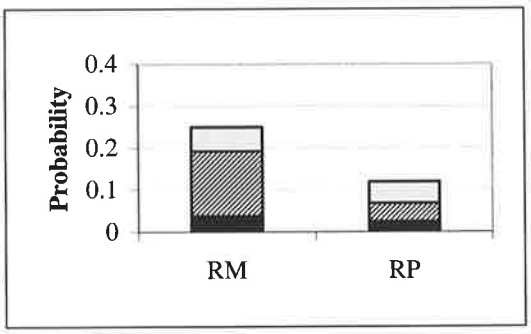
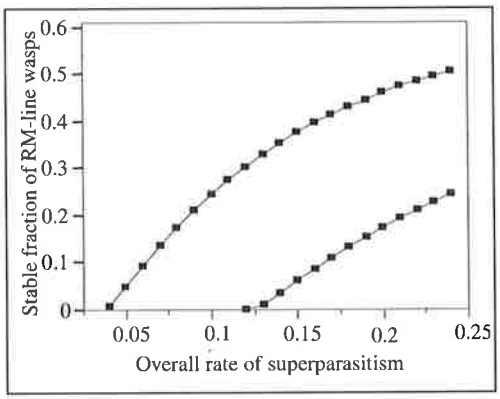
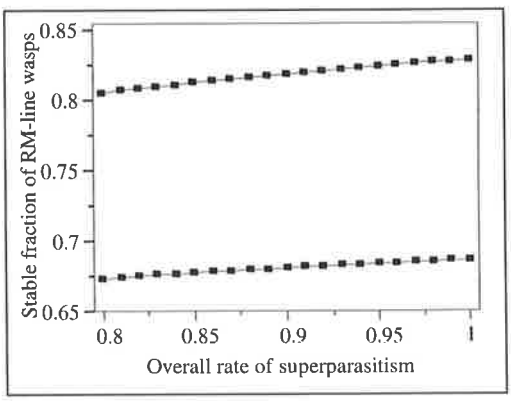


Figure 4. Predicted upper and lower boundary conditions for the relationship between the overall rate of superparasitism and the stable fraction of the RM-line in a mixed population of RM- and RP-line *V. canescens* under a) low levels and b) high levels of superparasitism. See text for details.

4a



4b



Chapter Eight

Genetic analysis of two distinct reproductive strategies in sexual and asexual field populations of an endoparasitic wasp, *Venturia canescens*

www.ucomics.com/calvinandhobbes/

STATEMENT OF AUTHORSHIP

Li, D.M., Zhao, Z., **Roberts, H.L.S.**, Schneider, M.V., Theopold, U. and Schmidt, O. (2003) Genetic analysis of two distinct reproductive strategies in sexual and asexual field populations of an endoparasitic wasp, *Venturia canescens*. *Heredity* **90**, 291-297.

Li initiated the experiments and together with Zhao performed the bulk of the experimental work. Roberts analysed the data, interpreted the results and with Schmidt co-wrote the paper.

Dongmei Li
Designed experiments, performed experimental work.

ZengQui Zhao
Performed experimental work.

Harry L. S. Roberts (Candidate)
Analysis and interpretation of data, co-wrote manuscript.

Maria Schneider
Assisted experimental work.

Ulrich Theopold
Assisted experimental work.

Otto Schmidt (Principal Supervisor)
Supervised work, co-wrote manuscript and acted as communicating author.

Signed (Harry Roberts)

.....*Date*.....

Signed (Otto Schmidt)

.....*Date*.....

Li, D., Zhao, Z., Roberts, H., Schneider, M. V., Theopold, U. & Schmidt, O. (2003). Genetic analysis of two distinct reproductive strategies in sexual and asexual field populations of an endoparasitic wasp, Venturia canescens. *Heredity*, *90*(4), 291-297.

Chapter Nine

Changes in a cluster of phenotypic characters in a strain of the endoparasitoid wasp *Venturia canescens* following alterations in culture conditions

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STATEMENT OF AUTHORSHIP

Roberts, H.L.S., Reineke, A. and Schmidt, O. (in preparation) Changes in a cluster of phenotypic characters in a strain of the endoparasitoid wasp *Venturia canescens* following alterations in culture conditions.

The observed incomplete penetrance of the RM-phenotype suggested the expression of the phenotype may involve a non-genetic determinant. The effects of a number of environmental factors were investigated, including host diet, oxygen partial pressure, parasitoid age at oviposition, and level of superparasitism. Only the latter resulted in alterations being observed. All the experiments were designed and performed by Roberts, with the exception that most of the molecular analyses were performed by Reineke.

Harry L. S. Roberts (Candidate)
Designed and performed experiments, analysed and interpreted of data, wrote manuscript.

Annette Reineke
Assisted with experimental work.

Otto Schmidt (Principal Supervisor)
Supervised work.

Signed (Harry Roberts)

.....*Date*.....

Signed (Otto Schmidt)

.....*Date*.....

Paper in preparation

Changes in a cluster of phenotypic characters in a strain of the endoparasitoid wasp *Venturia canescens* following alterations in culture conditions.

Harry L.S. Roberts*, Annette Reineke[1] and Otto Schmidt

The University of Adelaide, Insect Molecular Biology Laboratory, Glen Osmond SA 5064, Australia

[1] present address: University of Hohenheim, Institute of Phytomedicine, D-70593 Stuttgart, Germany

* corresponding author

email: harry.roberts@adelaide.edu.au

phone: +61-83037274

fax: +61-83794095

Keywords: RM, RP, reproductive success, egg maturation, superparasitism phenotypic plasticity

Abstract

Recent research has demonstrated that a long-standing laboratory culture of the asexual endoparasitoid wasp *Venturia canescens* contains two genetically distinct lines, coexisting on their host the flour moth *Ephestia kuehniella*. The two lines are addressed as RP (repeat plus) and RM (repeat minus) for the presence or absence of a 54 base-pair tandem repeat sequence in the gene coding for a virus-like particle (VLP1) protein. The lines are genetically stable, and differ in a range of phenotypic characters. Previous indirect evidence has suggested that the phenotypic differences between the lines are due to pleiotropic effects of the VLP1 alleles.

Here we report that maintaining the RM line under conditions of low superparasitism resulted in rapid alterations in the phenotypic characters calyx eggload, egg maturation rate, and reproductive success under single egg and under competing superparasitism. The observed changes were not associated with changes in the RM-VLP1 allele, and in general are difficult to explain in terms of genetic change.

Introduction

Endoparasitoid insects deposit their eggs inside the body of a host, exposing the larval stages of the developing parasitoid to the potentially fatal influence of the host's immune system. To facilitate the development of their young, many female endoparasitoids produce a range of ovarian and venom gland secretions that are injected into the host during oviposition (Beckage, 1997), where they interfere with the host's defense system and other host functions (Quicke, 1997). In the case of the solitary endoparasitoid wasp *V. canescens* these secretions include nucleic acid free virus-like particles (VLPs) that are produced in the calyx tissues of the reproductive tract (Feddersen *et al.*, 1986). In an asexual laboratory colony founded from wasps donated by George Salt in the 1960's, the DNA of one of the VLP proteins (coding for 40 kDa protein, named VLP1) was found to occur in two allelic variants, differing by the presence or absence of an 54 bp tandem repeat sequence (and named "repeat plus", RP and "repeat minus", RM). All wasps analysed were found to be homozygous for one of the two VLP1 alleles (Hellers *et al.*, 1996) and the genes were stable over successive generations (Beck *et al.*, 1999). Consequently, in combination with the population's asexual mode of reproduction, it was possible to establish two separate clonal lines based on the allelic variation. Once established, it was found that RM wasps produced fewer offspring than RP wasps. However, when the two strains were maintained as a mixed culture, RM wasps produced more offspring than the RP wasps (Beck *et al.*, 1999), and most interestingly, more offspring than they did as a pure culture. This suggests that the RM larvae gained some benefit from developing in a host that had been parasitised by an RP wasp.

Examination of the reproductive tracts of individuals from the two newly established lines revealed further differences. The calyx glands of RM wasps were larger than those of RP wasps, with extended VLP-filled membrane systems, and the amount of secreted VLP1 protein in the calyx was less for the RM wasps than the RP wasps. Differences were also observed in the transfer of eggs through the calyx to the oviduct, with 7-day-old RM wasps having significantly more eggs in the calyx and significantly fewer in the oviduct, compared to RP wasps (Beck *et al.*, 1999).

Reineke *et al.* (2003) investigated the reproductive success of the RM and RP lines under controlled inter-line superparasitism (= more than one egg oviposited into each host), with a wasp from each line allowed to oviposit a single egg each, with different time intervals between ovipositions. They found that the competitive abilities of the two lines were not symmetrical, with the RM-line winning a significantly higher fraction (around 60%) of the overall contests, probably due to an advantage of the RM-line in interlarval combat involving newly hatched larvae (Roberts *et al.*, 2004).

Examination of field collected sexual and asexual material from Australia and France showed the same cluster of traits associated with the RM and RP laboratory genotypes, albeit in a less pronounced form (Li *et al.*, 2003). This suggested that the different components of the laboratory RM phenotype shared a common underlying molecular cause.

However, the observed phenotypic differences in the morphology and eggload of the RP and RM calyx are not fully penetrant (Beck *et al.*, 1999; Li *et al.*, 2003), which suggests expression of the phenotype may involve a possible non-genetic determinant. Further, the higher absolute reproductive success of the RM line under inter-line, compared to intra-line, superparasitism (Beck *et al.*, 1999) suggests that the development of the RM larvae is beneficially affected by its host also being parasitised by an RP wasp.

This raises the question of whether the expression of the RM phenotype is modified by the degree of superparasitism and the genotype of the ovipositing adults. Known non-genetic mechanisms of phenotypic change in asexual lineages can be grouped into three overlapping categories, phenotypic plasticity, maternal effects and endosymbionts.

Phenotypic plasticity refers to how different phenotypes ("character states" (Schmalhausen, 1949)) can arise from a single genotype, as a result of different environmental conditions during ontogeny. While little is known about the genetic mechanisms that underlie adaptive phenotypic plasticity (Barton and Turelli, 1989), two general mechanisms have been identified. Allelic sensitivity refers to where the expression of an allele in different environments has varying effects on the phenotype. Gene regulation refers to the interaction between two genes, with a regulatory (epistatic) gene controlling the expression of the other (hypostatic) gene (Scheiner, 1993; Via, 1993).

Maternal effects include maternal imprinting, where there is a heritable change in gene expression without an actual change in DNA sequence, for example due to DNA methylation and 5-methylcytosine content (reviewed in Holliday, 1994)). There is also evidence that a character state induced in one generation can be transmitted to subsequent generations. For example, Rahman *et al.* (2004) showed that tolerance to a *Bacillus thuringiensis* formulation in the Phycitid moth *Ephestia kuehniella* correlated with an elevated immune response, which could be induced by pre-exposure to a low dose of the formulation and, once induced, transmitted to following generations through the female line.

Finally, it is known that infection by endosymbionts can result in profound phenotypic effects. For example, for many sexually reproducing wasps (but not including *V. canescens* (Beukeboom and Pijnacker, 2000)) infection by the bacteria *Wolbachia* results in a change to asexual reproduction, which is reversible if the infection is treated with antibiotics.

If the expression of the RM phenotype is influenced by the degree of superparasitism and the genotype of the ovipositing adults, then rearing the RM line under conditions of single egg parasitism would be expected to result in observable change. Here we report that culturing the RM line under conditions of low superparasitism resulted in rapid alterations in the phenotypic characters calyx eggload, egg maturation rate, and reproductive success under single egg and under competing superparasitism. The observed changes were not associated with changes in the RM-VLP1 allele, and in general are difficult to explain in terms of genetic change.

Method

Insects

The initial cultures of wasps (named RP (pure) and RM (pure)) were clonal RP- and RM-lines of *Venturia canescens* as described in Beck *et al.* (1999). After 70 generations of being cultured as isolated strains, a mixed RM/RP culture was established again (named RM (mixed)).

Rearing procedures

For general colony maintenance, wasps were reared in cylindrical clear plastic tubs; height 20cm and diameter 15cm. Four or five adult wasps were placed

into each container with 40-50 hosts until the wasps died (around 36 h). To determine the level of superparasitism under these conditions all hosts from one such container were dissected 5 days after set up. Upon emergence the wasps were removed from culture and kept in gauze-covered 425 ml clear plastic cup (Party Rite Jumbo Tumblers™, Harris Paper Pty. Ltd., West Heidelberg, Australia) with a 50% honey solution. Hosts were *Ephestia kuehniella,* reared on a 10:2:1 mixture of oat bran, wheat germ, and dried brewers yeast. All experiments were conducted and cultures maintained at 25 ± 1°C, under a constant light-dark regime (L14:D10).

Singly parasitised hosts were obtained using the method of Roberts *et al.* (2004). For large scale culturing of the RM line under conditions of limited host access, single RM wasps were given access to 20 hosts for one h. Wasps from the RM (pure) line were reared under conditions of strict single parasitism for one generation (described as RM (pure)s) and then for a further 5 generations under conditions of limited host access. There were 20 replicate lines. The replicate lines were then pooled and the new colony (labelled RMN for RM new) was used to assess a range of phenotypic characters. Generation numbers for the RMN line are counted from the generation the replicate lines were pooled. To determine the level of superparasitism under these conditions all hosts from five such containers were dissected 5 days after set up.

Measurement of phenotypic characters

Measurement of adult wasp size and molecular genetic analyses were performed as per Reineke *et al.* (2003). Calyx eggload and total eggs matured by 7-day-old host-deprived wasps were determined as per Roberts and Schmidt (2004). Reproductive success under single and inter-line superparasitism was determined as per Roberts *et al.* (2004).

Statistical analysis

Data were analysed using the generalised linear model (GLM) platform, JMP V4.0.4 (SAS Institute Inc., 2001), with continuous factors centred by their means (Neter *et al.*, 1990). Analyses started with full models with all interactions, and interactions were progressively dropped if $P>0.25$ (Winer, 1971).

Results

Levels of superparasitism under colony maintenance and limited host access conditions. Excluding unparasitised hosts from the analyses, under limited host access conditions 82% of hosts contained a single egg while under colony maintenance conditions the mean number of eggs per host was 6.4, with no host containing fewer than three eggs (Fig. 1).

VLP typing of the lines. Analysis of the VLP1 proteins of the lines by western blots confirmed that the RM and RMN lines were homozygous for the RM-VLP1 protein and the RP line was homozygous for the RP-VLP1 protein.

Calyx eggload. There were significant differences ($F=29.3$, $df=6,641$, $P<0.0001$) in the numbers of eggs in the calyx regions among the 3 lines (Table 1). *Post hoc* analysis (Tukey-Kramer, $\alpha=0.05$) showed that the differences between the F10 and *ca*F80 generations of the RM (pure) line, the *ca*F20 generation of the RM (mixed) line and the offspring under single parasitism of the *ca*F80 RM line were not significant. Similarly, there was no significant difference between the F2 and F10 generations of the RMN line. In contrast, significantly fewer eggs were present in the calyx region of the RMN wasps compared to the various RM wasps.

Eggs matured by 7-day-old host-deprived wasps. There were significant differences ($F=26.7$, $df=2,132$, $P<0.0001$) in the total number of eggs matured by 7-day-old host-deprived wasps between the RP, RM (mixed) and RMN lines (Fig. 2). Analysis by Tukey-Kramer LSD ($\alpha=0.05$) indicated that the RMN line matured significantly fewer eggs than either the RM (mixed) or RP lines.

Reproductive success under single egg parasitism. There were significant differences in the number of offspring from hosts between the RM, RMN and RP lines (Table 2). Pair-wise tests showed that the difference between the RM (pure) and RM (mixed) cultures was not significant. Similarly, the difference between the RP and RMN lines was not significant while the RM lines produced significantly fewer offspring under single parasitism compared to both the RP and RMN lines.

Reproductive success under inter-line superparasitism. Analysis of the outcome of inter-line superparasitism (RM vs RP, Fig. 3a and RMN vs RP, Fig. 3b) with time interval between ovipositions and RM line (RM coded as 1,

RMN coded as 2) as factors revealed that the RM line produced significantly more offspring (X^2=43.6, df=2, P<0.0001) than the RMN line when in competition with the RP line. There was also a significant interaction between RM line and time (X^2=14.1, df=2, P=0.0009).

Discussion

While a high level of superparasitism is the norm under standard colony maintenance conditions, with an average of 6.4 eggs per host, single egg parasitism is the norm under the limited host access condition, with only 18% of hosts containing more than one egg. When the RM wasps were maintained as either pure or mixed cultures under conditions of high superparasitism, the expression of the phenotype, as indicated by the calyx eggload, did not change significantly over time. Similarly, there was no significant change in calyx eggload in the first generation of RM wasps reared under conditions of single egg parasitism (RM (pure)s). However, following a further five generations of rearing under conditions of predominantly single egg parasitism, the phenotype expressed by the RM line (now described as RMN, for RM new) showed significant differences to the other RM cultures in all four of the characters examined. Compared to the RM (pure) line, the RMN line retained fewer eggs in the calyx region, had a higher rate of reproductive success under single parasitism and a lower rate of reproductive success under inter-line superparasitism with the RP line. Similarly, compared to the RM (mixed) line, the RMN line retained fewer eggs in the calyx region, and also showed a lower rate of egg maturation under conditions of host deprivation. In contrast, there were no significant differences between the RMN and RP lines in the numbers of eggs in the calyx region, or in reproductive success of the two lines under single parasitism or competitive superparasitism. Further, after the RMN line was cultured under conditions of superparasitism for a total of 10 generations no significant change to the calyx eggload phenotype was observed.

Since the *Venturia* lines in the study are asexual, these results raise the question of how the observed differences in the RM phenotype can occur in only a few generations.

The two simplest explanations, either contamination of the RM colony by a third strain or selection for a pre-existing RMN genotype are difficult to imagine for a number of reasons. Any contamination would be expected to have a 50% chance of being by a wasp of the alternative VLP1 allele. The pure colonies had been maintained collectively for around 150 generations without alternative VLP1s being observed, suggesting that contamination, if it has occurred at all, is a rare occurrence. Further, there was no significant change in the calyx phenotype between the 10^{th} and 70^{th} (immediately prior to the change in culturing conditions) generations of the RM (pure) line, indicating that the composition of the culture had not changed in that time. Contamination or selection as the basis is also unlikely due to the nature of the breeding program. Since each founder wasp generated an independent line, there was no competition for hosts between the offspring of the different females and hence no selection pressure relating to reproductive success under single egg parasitism. Thus, the fraction of the population represented by a putative pre-existing RMN genotype should not change over the five generations that the wasps were reared under conditions of limited host access.

Explanations based on genetic change are also implausible. Possible genetic change in asexual strains can occur through a number of processes, such as crossing over and mutation, including microsatellite slippage. In asexual *V. canescens*, the mode of reproduction can be categorized as central fusion automictic parthenogenesis (Beukeboom and Pijnacker, 2000), with two haploid pronuclei that segregated at meiosis I fusing, following meiosis II, to restore diploidy. Since crossing over can occur prior to meiosis I, over successive generations the degree of heterozygosity in a lineage will decrease the more distal the loci are to the centromere, so that a group of siblings may be genetically different from both their mother and each other. Thus, while in principle evolutionary adaptation in asexual *V. canescens* lineages should be possible from existing genetic diversity, it will likely be in a limited and possible irreversible form (Slobodchikoff, 1983). Phenotypic changes can also be based on mutational genotype alterations. Although there are reports on increased mutational rates in hymenopteran parasitic wasps (Dowton and Austin, 1995), it is difficult to reconcile a phenotypic

change based on a general increase in mutation rate with the otherwise normal fitness observed in both strains. An increase of mutations in localised areas of the genome is possible, particularly if the mutations are in DNA 'hot spots', such as microsatellites that can expand and contract by DNA-polymerase slippage mechanisms. However, invoking genetic change based on selection of spontaneously arising mutants implies similar changes of the independent lines, which has not been observed.

If explanations based on contamination, selection for a pre-existing genotype or genetic change are rejected, then the simplest explanation, as suggested by Amat *et al.* (2003), is that the RM phenotype is due to a pathogen, specifically, an interaction between the RM genotype and a semi-permissive endosymbiont, where transmission is vertical via maternal secretions from adult wasp to host and then horizontal from host to larval parasite. Thus, if the level of superparasitism is high, then the maternally derived endosymbiont load, and hence the probability of a wasp larva being infected, are also high. In contrast, under predominantly single parasitism the probability of transmission is low and so over successive generations the endosymbiont is lost.

The finding that the RM phenotype was expressed in the first generation of larvae reared under single egg parasitism, but then lost over the subsequent generations, suggests that the phenotype of the offspring depends at least in part on the maternal phenotype. While this does not preclude infection by an endosymbiont from being the cause, it suggests a possible alternative, where the RM phenotype emerges due to an interaction, mediated by the host, between the RP maternal secretions and the developing RM wasp. Once the RM phenotype has emerged it is maintained under conditions of high intra-RM-line superparasitism (and hence high levels of maternal secretions) by maternal transmission, but lost over successive generations under conditions of single egg parasitism (and hence low levels of maternal secretions). Such a system may be functionally similar to one described by Rahman *et al.* (2004), where exposure of larval *E. kuehniella* to sublethal concentrations of a *Baccillus thuringiensis* toxin formulation led to an elevated immune response which could be transmitted to offspring by a maternal effect.

Irrespective of the basis, the finding that culture conditions affect the

expression of a cluster of phenotypic characters in a strain of the endoparasitoid wasp *V. canescens* has profound implications on the ecology of the wasp in the field. The induction of phenotypic traits and their transmission to subsequent generations can create distinct phenotypes in subpopulations that are genetically identical. This is particularly relevant in parasitoid populations that are reared under laboratory conditions to be released into the field as biological control agents.

Acknowledgements

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Figures

Figure 1. Distribution of the number of eggs deposited by *V. canescens* in each *E. kuehniella* larva: **black bars** colony maintenance conditions (4 wasps given access to 50 hosts for 24h), **grey bars** limited host access conditions (single wasps given access to 20 hosts for one h).

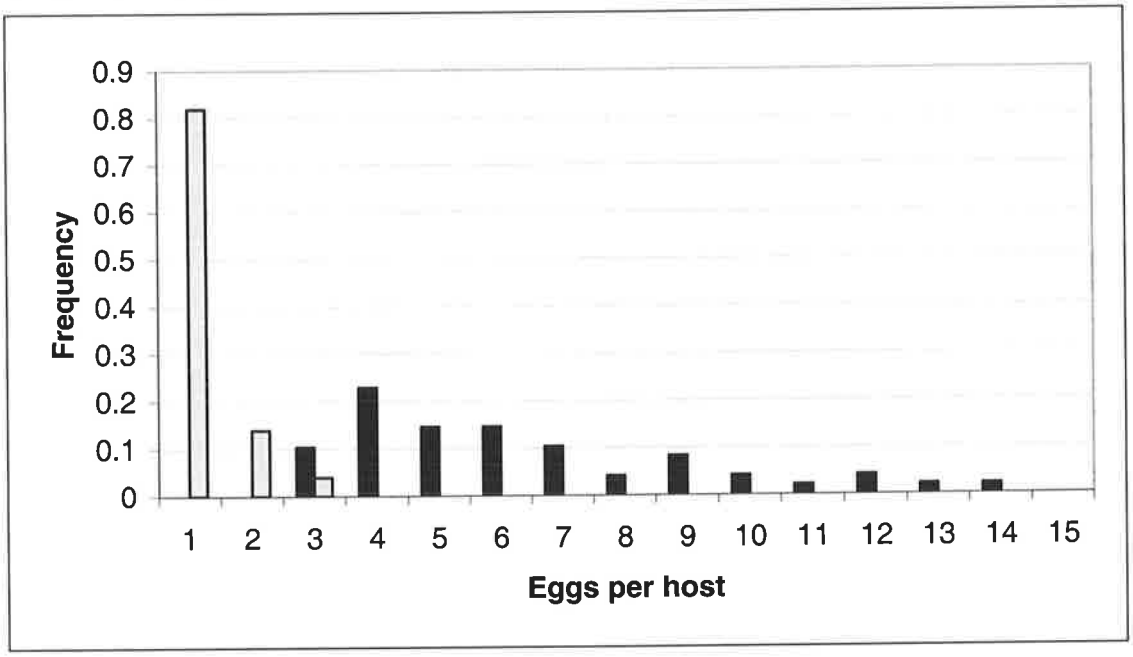


Figure 2. Relationships for wasps from three lines between *V. canescens* head capsule width in mm, and total number of eggs matured by wasps deprived of hosts for seven days, Triangle and solid line is RP (Roberts *et al.*, 2004), closed circle and dotted line is RM (mixed), grey diamond and dashed line is RMN.

Generation number, and least squares means and SE of total number of eggs matured are shown in the box. L S means with the same letter were not significantly different (Tukey-Kramer LSD, α=0.05).

Line	Generation	L S mean	SE
RMN	2	203.1 **a**	3.1
RM (mixed)	*ca* 20	231.1 **b**	3.6
RP	*ca* 80	232.4 **b**	3.2

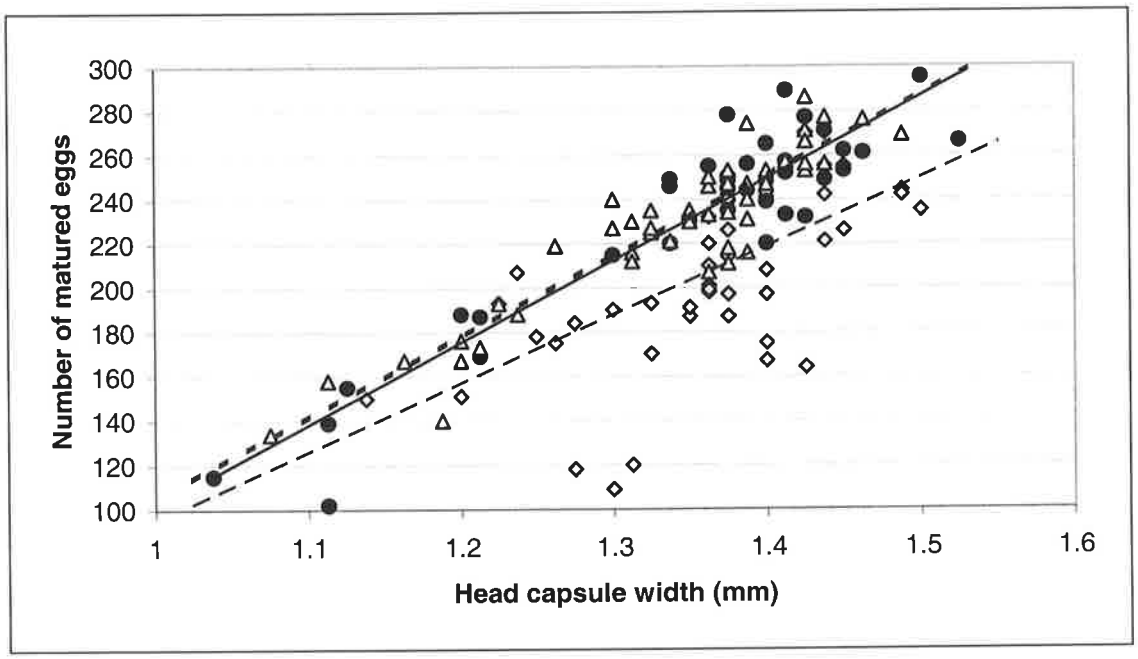
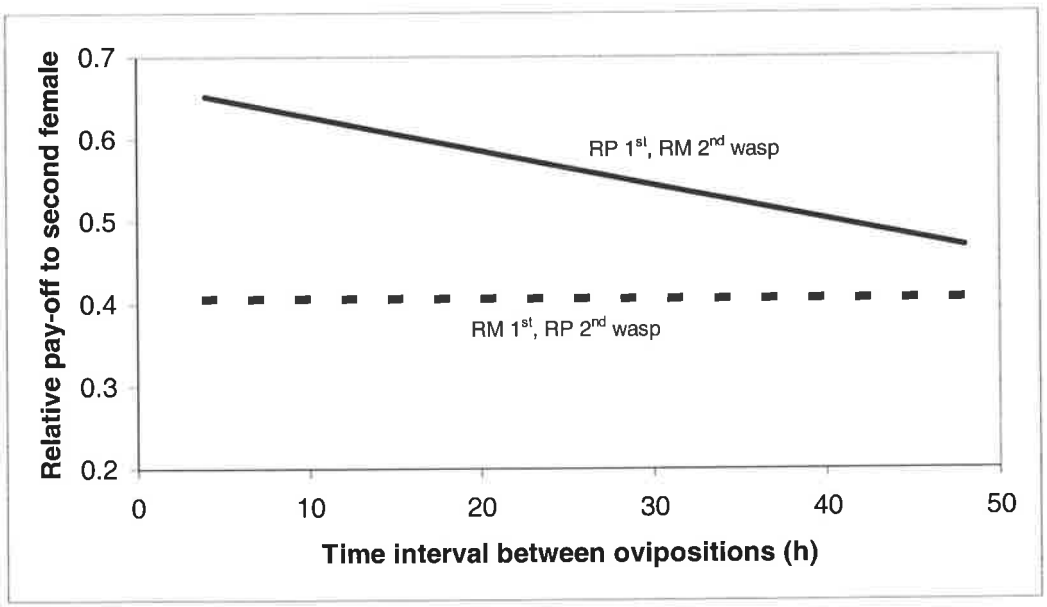
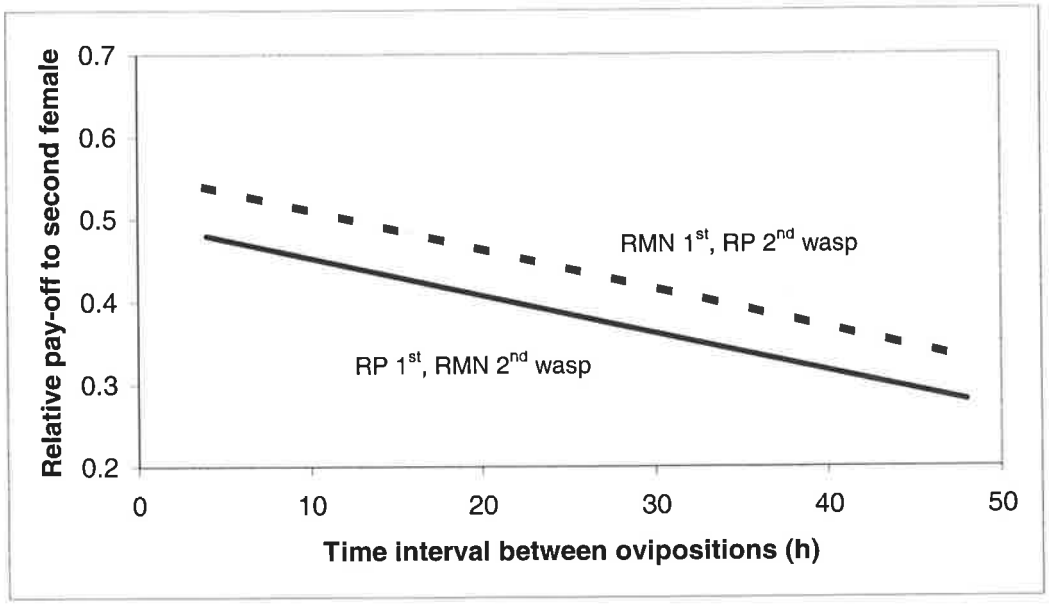


Figure 3. The fraction of emergent wasps that were the offspring of the superparasitising female (the relative pay-off from superparasitism), as a function of the time interval between ovipositions for the two different orders of oviposition. Lines represent the fitted values from the logistic regression model. **a** 'RM pure' first, 'RP' second (dashed line) and 'RP' first, 'RM pure' second (solid line). (Reineke *et al.*, 2003), **b** 'RMN' first, 'RP' second (dashed line) and 'RP' first, 'RMN' second (solid line). (Roberts *et al.*, 2004).

3a



3b



Tables

Table 1. Number of eggs in the calyx region of 7-day-old host-deprived *V. canescens*. Means with the same letter were not significantly different (Tukey-Kramer, α=0.05).

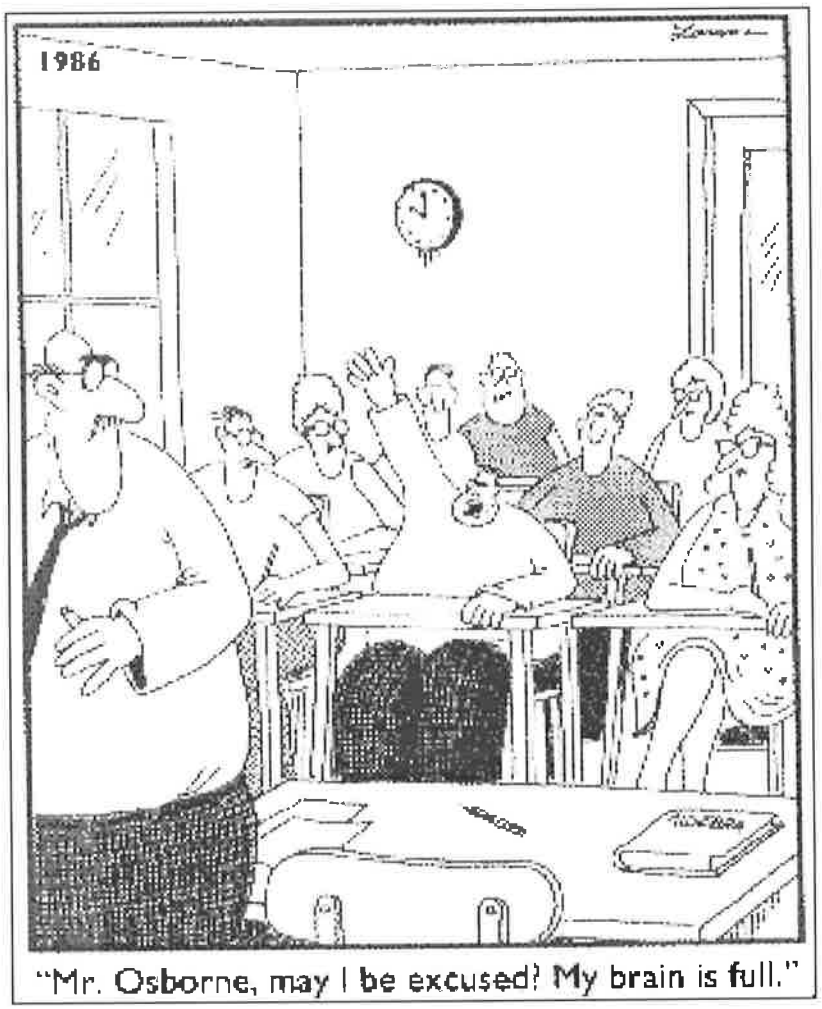
Line	Generation	N	Mean	95% CI
RM (pure)	*ca* 10	168	13.6 **a**	12.0 - 15.2
RM (pure)	*ca* 80	114	11.5 **a**	8.7 - 14.4
RM (pure)s	*ca* 80+1	44	12.7 **a**	9.1 - 16.3
RM (mixed)	*ca* 20	72	15.4 **a**	10.2 - 20.6
RMN	2	102	0.9 **b**	0.5 - 1.3
RMN	10	50	0.8 **b**	0.5 – 1.1
RP	*ca* 80	98	0.7 **b**	0.1 - 1.3

Table 2. Percentage *V. canescens* offspring from *E. kuehniella* larvae under conditions of controlled single egg parasitism. Means with the same letter were not significantly different (X^2 test, α=0.05).

Line	Generation	N	% offspring	95% CI
RM (pure)	*ca* 80	74	71.6 **a**	60.5 - 80.6
RM (mixed)	*ca* 20	156	75 **a**	67.7 -81.1
RMN	2	103	86.4 **b**	78.5 - 91.7
RP	*ca* 80	117	88 **b**	80.9 - 92.7

Chapter Ten

Synthesis and Discussion



Gary Larson and Steve Martin, (2003) *The Complete Far Side*. Andrews McMeel Publishing, New York

It has recently been shown that a thelytokous laboratory culture of the parasitoid wasp *Venturia canescens* contains two genetically distinct lines, coexisting on their host the flour moth *Ephestia kuehniella* (Hellers *et al.*, 1996; Beck *et al.*, 1999, 2000b, 2001). The two lines differ genetically by the presence (the RP-line) or absence (the RM-line) of a 54 base-pair tandem repeat sequence in the gene coding for a virus-like particle (VLP1) protein (Hellers *et al.*, 1996). The lines are genetically stable, and differ in a range of phenotypic characters, including ovarian morphology, calyx gland secretions and reproductive success (Beck *et al.*, 1999, 2000b, 2001). The laboratory culture had been maintained without the addition of new stock for over 400 generations, suggesting, in apparent contradiction of the principle of competitive exclusion (Gause, 1934; Volterra, 1926) that the two lines had been coexisting sympatrically while competing for the same resource.

The primary aim of the research presented in this thesis was to determine the basis of the coexistence of the two wasp strains, and estimate whether it is an artefact of the laboratory rearing conditions, or could in principle occur in field populations.

Controlled oviposition experiments showed that the RM-line won a significantly higher fraction (around 60%) of the overall contests, and further, that the competitive abilities of the two lines were not symmetric, indicating that the advantage of the RM-line related to one-on-one interlarval competition rather than differences in maternal behaviour. Dissection of parasitoid larvae from superparasitised hosts indicated that most contests between competing larvae had occurred within the first 24 h of the eggs hatching, suggesting the advantage of the RM-line relates primarily to physical combat rather than to physiological suppression (Reineke *et al.*, 2004).

Investigations of interlarval combat under *in vitro* conditions showed that the outcome depended on both the relative and absolute ages of the contestants, and confirmed that the competitive abilities of larvae from the two lines were not symmetric. The higher reproductive success of the RM line under conspecific superparasitism was found to correspond to a physiological difference between the newly hatched larvae of the two lines which results in

an advantage to the RM larva independent of the order or time interval between ovipositions (Roberts *et al.*, 2004a).

Dissection of singly-parasitised hosts in which parasitism had failed indicated that the lower rate of parasitism success for the RM-line when reared alone is due to an increase in the rate of failure as late instars. There was no evidence that larvae had succumbed to a melanisation or encapsulation response, and so the cause of failure is unclear (Roberts *et al.*, in press).

An analysis was conducted of the influence of host mass and the time interval between ovipositions on the survival and development of larvae from both the first and second laid eggs in superparasitised *E. kuehniella*, in this case involving two competitively similar lines. It was found that as the time interval between ovipositions increased both overall and superparasitism success decreased, however time between, and order of, ovipositions had little effect on other developmental parameters. Adult size increased with host mass under both parasitism and superparasitism, while host mortality decreased with host mass under superparasitism. In addition, wasps emerging from superparasitised hosts were larger than wasps from parasitised hosts (Roberts *et al.*, 2004b).

Research on the lifetime fecundity of female *V. canescens* revealed the previously unreported phenomena of egg dumping by an endoparasitoid wasp when deprived of hosts. Female *V. canescens* maintained without hosts began to deposit eggs onto the sides of the culture vessel on the day of eclosion. The maturation of additional eggs was not inhibited once the maximum oviduct egg load was reached but rather continued for the duration of the experiment (up to 39 days), at a rate of around 5.8% of the remaining unmatured eggs per day (Roberts and Schmidt, 2004).

Following this research an iterative mathematical model was developed that uses a range of experimental life history data to predict the stable composition of a mixed population of two lines displaying the laboratory phenotypes under different rates of superparasitism. The model predicts that sympatric coexistence of the two lines is possible when the overall rate of superparasitism is between 4 and 12% or greater. These values are within the rates reported for other solitary endoparasitoid wasp species in the field, and

so demonstrate that the sympatric coexistence under natural conditions of two species that display the phenotypes observed in the laboratory lines is, *in principle*, possible (Roberts *et al.*, in press).

However, the question addressed by the secondary aim of the research, whether the phenotype observed in the laboratory RM-line is due to pleiotropic effects of the allelic VLP1 gene locus, remains unresolved.

Examinations of four pairs of field-derived lines have found that the traits variously associated with the RM and RP laboratory genotypes cluster together, typically in a less pronounced form (Amat *et al.*, 2003; Li *et al.*, 2003b; Roberts *et al.*, 2004b). This correspondence suggests that the different components of the laboratory RM phenotype share a common underlying physiological cause, while the reduced level of expression suggests two different factors can potentially be involved. Further, the expression of the phenotype is not fully penetrant (Beck *et al.*, 1999; Li *et al.*, 2003b), and can change with culture conditions (Roberts *et al.*, in preparation), which suggests expression of the phenotype may involve a non-genetic determinant.

Within these parameters two general explanations of the RM-phenotype have been proposed, that the RM-phenotype is either due to an endosymbiont or pathogen acting in some unknown fashion (Amat *et al.*, 2003), or due to pleiotropic effects of the allelic VLP1 gene locus (Beck *et al.*, 2000b, 2001).

The key components of the RM-phenotypic cluster of traits are: calyx membrane alterations leading to the development of extensive luminal membrane systems and the retention of eggs in the calyx region (Beck *et al.*, 1999), the superiority of RM-larvae over RP-larvae in fighting ability for the first few hours after hatching (Roberts *et al.*, 2004a), and an increased probability that parasitism by RM wasps alone will fail at the late larval/ prepupal stage (Roberts *et al.*, in press).

Several lines of evidence support the pathogen model. Amat *et al.* (2003) examined two field-derived strains homozygous for the RM- and RP-VLP1 and found that they both displayed traits of the RM-phenotype. This implies that the RM-VLP1 is not necessary for the expression of the RM-phenotype. Further, Roberts *et al.* (in preparation) found that following five

generations of predominantly single parasitism the phenotype of the laboratory RM-line changed to a predominantly RP-line phenotype. This result is difficult to account for in terms of genetic change, and so the simplest explanation is that it is due to the loss of an endosymbiont or a pathogen.

What is the possible mode of action of a pathogen? It is conceivable that the RM-calyx membrane alterations arise in some fashion through the effects of an endosymbiont or pathogen developing in the calyx gland region. In an analogous fashion to that described later, this in turn could give rise to the competitive superiority of RM-larvae, through alterations in the concentration of calyx secretions co-injected with the egg into the host.

The increased rate of failure of RM-parasitised hosts at the late larval/ prepupal stage is perhaps the easiest of the traits to explain under a pathogen model. It has been found, for a range of pathogens, that when a parasitoid grows inside an infected host developmental failure can occur at the pupal or pharate stage, depending on the time interval between parasitism and infection (eg King and Bell, 1978; Temerak, 1980; Santiago-Alvarez and Caballero, 1990). Further, parasitism can lead to an increase in the host's resistance to a subsequent infection (Begon *et al.*, 1999). If the resistance of the host is due to the effects of a calyx secretion acting globally, and a consequence of the RM calyx tissue alteration is a general reduction in the concentration of maternal secretions, then RM-parasitised hosts may be more susceptible to infection and eventual failure.

There are a number of difficulties with the endosymbiont or pathogen model. Differential ovarian DNA expression of the laboratory RM- and RP-lines using cDNA-AFLP analysis (Reineke *et al.*, 2003) revealed a number of differences, one of which was shown to be a picorna-like virus of the genus *Ilfavirus* (Reineke and Asgari, in press). However, analysis revealed the virus was also present in the laboratory RMN-line (Roberts, unpublished data) and not present in either of a pair of other lines, homozygous for the RM and RP VLP1 gene and displaying the respective phenotypes (Li *et al.*, 2003b); (Reineke and Asgari, in press). Thus, it is unlikely the *Ilfavirus* is responsible for the phenotype, and Reineke *et al.* (2003) detected no other pathogens. The possible mode of transmission of a putative endosymbiont poses further difficulties for an explanation based solely on the action of a pathogen. If

transmission is strictly vertical, then it should not be affected by culture conditions. Alternatively, if transmission were vertical to the host and then horizontal from the host to the larval parasitoid, then under conditions of competitive superparasitism it would be expected to be present in both the RP- and RM-lines. Thus, the pathogen model would seem to require the phenotype be due to an interaction between the pathogen and the laboratory RM-genotype.

The alternative explanation is that the RM-phenotype is due to pleiotropic effects of the RM-VLP1 and at least one other factor, through a reduced capacity to control the effects of oxidative activity. A number of lines of evidence support this hypothesis, although it too is not without its difficulties.

The VLP1 protein shows sequence similarities to vertebrate PHGPxs (Hellers *et al.*, 1996), enzymes involved in the reduction of oxidised phospholipids. This suggests that a possible function of the VLP1 is to reduce oxidised lipids in membranes. While the VLP1 does not show enzymatic activity (Li *et al.*, 2003a), it may still bind to oxidised phospholipids and mask any deleterious or elicitor effects. The latter function is more likely since enzymatic activity would require the presence of reduced glutathione, which is unlikely to be available in extracellular locations such as the calyx lumen. Evidence also suggests that *V. canescens* calyx fluid has serine protease inhibitor (serpin) activity and is able to inhibit the activation of the melanising enzyme phenoloxidase (PO) (Beck *et al.*, 2000a).

As discussed in Schmidt *et al.* (in press), observations indicate that *V. canescens* oocytes and eggs contain PO-like enzymes, and are a source of reactive electrons that have the potential to damage the membranes of calyx tissues. The structural changes from the deletion of the tandem repeat sequence in the RM-VLP1 may reduce the capacity of the protein to bind membrane lipids oxidised by the reactive electrons. Reduced particle-membrane interactions and/or exposure of oxidised phospholipids may lead to the VLP-membranes merging with each other. Once these membranes start to form they may act as a positive feedback loop of ever increasing oxidative damage: the membranes progressively reduce the secretion of additional calyx fluid, including the VLP1 and the putative serpins (Beck *et al.*, 2000a),

resulting in an increase in reactive electron species and a decrease in capacity to repair damage, and thus over time leading to the formation of the observed extensive damage to calyx tissues and the creation of luminal membrane systems. Once formed, these constitute an obstacle to the passage of eggs, leading to the observed retention of eggs in the calyx region.

Another consequence of the reduction in the amount of calyx proteins secreted into the calyx lumen (Schmidt *et al.*, in press) would be a reduction in the amount of VLPs and calyx fluid that surrounds the egg when it is injected into the host. It is plausible that the superiority of RM-larvae over RP-larvae in fighting ability, given it only extends for the first few hours after hatching, is due to the mandibles or cuticle of the RM-larvae being relatively more sclerotised at the time of hatching (Roberts *et al.*, 2004a). Calyx fluid possesses serpin activity (Beck *et al.*, 2000a) and this activity is reduced in RM-calyx fluid (Beck, 1998), which suggests that RM-line eggs and embryos may be exposed to a higher level of host PO activity. This could result in higher levels of sclerotisation in the newly hatched RM-larvae, either directly by enhancing tanning of the RM-larval cuticle, or indirectly by strengthening the egg shell so that hatching is delayed (Beck *et al.*, 2001), allowing more time for the normal cuticular tanning processes to occur.

While the VLP proteins are secreted in greatest concentration in the calyx region, they are known to be present at lower but significant levels in a number of other tissues as well, including in the gut of the larval parasitoid (Kinuthia, 1996). This suggests a mechanism for the third key component of the RM-phenotypic cluster, an increased probability that parasitism by RM wasps will fail at the late instar/ pre-pupal stage. *V. canescens* does not appear to engage in systemic suppression of the host's immune response, but rather, relies on molecular mimicry and localised immune protection at the host/parasitoid interface (Kinuthia *et al.*, 1999; Schmidt *et al.*, 2001). Larvae are exposed to the host's immune response in two areas, the external cuticle and the gut. There is no evidence to suggest that larvae of either line are attacked by an external encapsulation response unless they have previously been wounded during interlarval combat. However while the external cuticle is a containing barrier, the gut must allow the uptake of nutrients from the

ingested host haemolymph. This will contain active host immune components including prophenoloxidase that is potentially activated by gut proteases, producing reactive electron species. Thus the gut would require some form of immune protection. This would plausibly involve at least two components: one to inactivate PO activity, and a second to repair oxidative damage. This is analogous to the situation in the calyx, and so it is tempting to speculate that the parasitoid employs a similar solution, serpins to inhibit the production of reactive oxygen species and the VLP1 to mask or remove any oxidative damage. Consistent with this hypothesis, Kinuthia (1996) observed that anti-VLP antibodies (i.e. antibodies raised against a preparation containing all the different VLP proteins) stained both the lining and, to a lesser extent the contents, of the gut lumen. In addition, preliminary Western blots of parasitoid gut contents stained with anti-PPO antibodies found that RP-larval gut contents showed a number of strong lower molecular weight bands indicative of PO breakdown products, while RM-larval gut contents showed much weaker lower molecular weight bands (Roberts, unpublished data). However, no differences were observed between blots of haemolymph from RM- and RP-parasitised hosts, suggesting the difference is due to larval gut secretions rather than systemic suppression of host haemolymph immune factors.

Together, these observations suggest a model where, by a mechanism analogous to that in the calyx, the lower capacity of the RM-VLP1 to bind oxidised membrane lipids results in increasing levels of damage to the gut lining, in turn reducing the amount of proteins being secreted. If the level of secreted proteins falls below some threshold required to control the host-derived immune active components of the gut contents then the gut contents coagulate and the parasitoid dies. Noting that Kinuthia (1996) observed from UV visualisation of parasitoid gut tissue sections that the transition from late larval to pre-pupal stages was associated with an increase in necrotic cells, presumably as the internal structure of the gut changes in preparation for adult life, and that this corresponds to the stage at which the rate of RM-parasitism failure is greatest (Roberts *et al.*, in press), it is plausible that the onset of the transition represents the point where control is most likely to be lost.

An implication of this general model is that RM-larvae develop in hosts containing lower quantities of maternally derived calyx proteins. However, apart from an increase in the rate of parasitism failure of the RM-line under non-competitive parasitism, no major differences in larval development have been observed. Is this consistent with the model? Two lines of reasoning suggest that it is.

To be successful the parasitoid must be protected from the host's immune system and manipulate the host's physiology. As discussed in Roberts *et al.* (2004b), it is likely that the task of manipulating the physiology of the host is performed by both maternal and larval secretions. The available evidence suggests a two-step process, where one of the roles of maternal secretions is to inhibit host control of physiological processes, while the secretions of the larva manipulate the processes to facilitate the parasitoid's own development. The maternal secretions comprise the calyx fluid and the venom gland secretions. The apparent functions of all the calyx fluid components analysed to date have related exclusively to providing protection against the host's immune response (Asgari *et al.*, 2002; Beck *et al.*, 2000a; Li *et al.*, 2003a; Reineke *et al.*, 2002). This implies that it is probably venom gland secretions that perform the role of inhibiting host control of physiological processes. Consistent with this suggestion, unparasitised *E. kuehniella* that were injected with a volume of whole calyx fluid equivalent to being parasitised around 20 times showed no differences in mortality or development compared to untreated controls (Roberts, unpublished data). Since there is no reason to suspect systematic differences in the amount of either venom gland or exogenous larval secretions, it follows that there is no reason why the two strains should differ in their capacity to control the physiological processes of the host. Further, the model posits that parasitism failure in the RM-line is not due to the level of gut membrane damage impacting on nutrient uptake so that the parasitoid starves, but rather that parasitoid death occurs at a late stage from a failure to control an immune response in ingested host haemolymph, and thus would not predict major differences in larval growth and development between the two strains

Another finding that requires explanation is that expression of the RM-phenotype is variable between different strains (Li *et al.*, 2003b). The essence

of the general model is an interplay between two processes, the generation of reactive electron species and the repair or masking of their effects. If it is assumed that the VLP1 is the key component in the repair/masking process, then two potential explanations of the between strain variability can be envisioned. First, the observed serpin activity may be due to multiple peptides and/or alleles that vary in their effectiveness (Beck *et al.*, 2000a), so that the level of reactive electron species varies from strain to strain. Alternatively, the serpins may be differentially expressed in the different strains, for example with a strain in which the serpin-coding genes are up regulated and hence display a reduced RM-phenotype. Likewise, a variation in calyx phenotypes may be due to variation in the oxidative sources (copper-containing chorion proteins) or in the repair/masking of oxidised lipids and proteins by the VLP1 protein.

The latter option also provides an in principle explanation for the observation that maintaining the laboratory RM-line under conditions of low superparasitism resulted in rapid alterations in calyx morphology and reproductive success under single egg parasitism compared to competing superparasitism. These changes were not associated with changes in the RM-VLP1 allele, and in general are difficult to explain in terms of genetic change (Roberts *et al.*, in preparation). If we assume that the expression of the RM-phenotype can vary with the relative up or down regulation of the ovarian proteins, then all that is required is that the expression levels can be modified by environmental factors that are able to be transmitted in a non-genetic manner from mother to offspring. The model involves the RM-phenotype emerging due to an interaction, mediated by the host, between the RP maternal secretions and the developing RM wasp. Once the RM phenotype has emerged it is maintained under conditions of high intra-RM-line superparasitism (and hence high levels of maternal secretions) by maternal transmission, but lost over successive generations under conditions of single egg parasitism (and hence low levels of maternal secretions). Such a system may be functionally similar to one described by Rahman *et al.* (2004), where exposure of larval *E. kuehniella* to sublethal concentrations of a *Bacillus thuringiensis* toxin formulation led to an elevation of the immune response which could be transmitted to offspring by a maternal effect and

maintained in the absence of the external elicitor.

The above discussion seeks to show how the observed phenotypic data can be adequately explained in terms of what is currently known of the insect's molecular biology. However it must be acknowledged that other explanations are possible. Once the remaining components of the calyx secretions have been identified and characterised, the detail of the model may well change. As noted earlier, the model is not without its difficulties. While the model does not invoke any processes that have not been demonstrated to occur in other systems, a number of the elements of the model are not supported by any direct experimental evidence. For example, the serpins in the calyx fluid have not been identified (Beck *et al.*, 2000a), nor have the putative gut secretions. Also, it is very difficult to explain the observation by Amat *et al.* (2003) of a homozygous RP-line line displaying RM-phenotypic traits. However, given the studies demonstrating that the RM-phenotype has a relative competitive advantage under conditions that favour superparasitism (Beck *et al.*, 1999; Reineke *et al.*, 2004), it would not be surprising if strains displaying different pathways to the RM-phenotype occur in field populations. Nor would it be surprising if the eventual explanation of the RM-phenotype included elements of both the pathogen/endosymbiont and allelic VLP1 models.

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Roberts, H.L.S., Keller, M. and Schmidt, O. An empirical model of the sympatric coexistence of two strains of the endoparasitoid wasp *Venturia canescens*. *Archives of Insect Biochemistry and Physiology*.

Schmidt, O., Li, D., Beck, M., Kinuthia, W., Bellati, J. and **Roberts, H.L.S.** Phenoloxidase-like activities and the function of virus-like particles in ovaries of the parthenogenetic parasitoid *Venturia canescens*. *Journal of Insect Physiology*.

Appendix One.

Other publications by the candidate

During my candidature I was involved in several other projects, that were not related to the investigation of the sympatric coexistence of the two strains of *V. canescens,* but which led to a number of other publications. These papers are presented in this appendix, in order to give a full representation of the work I performed during my time as a PhD candidate.

Appendix 1A.

Phenoloxidase-like activities and the function of virus-like particles in ovaries of the parthenogenetic parasitoid Venturia canescens.

Schmidt, O., Li, D., Beck, M., Kinuthia, W., Bellati, J. and **Roberts, H.L.S.** (in press) *Journal of Insect Physiology*.

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Phenoloxidase-like activities and the function of virus-like particles in ovaries of the parthenogenetic parasitoid *Venturia canescens*

Otto Schmidt [a,*], Dongmei Li [b], Markus Beck [c], Wanja Kinuthia [d], Judy Bellati [a], Harry L.S. Roberts [a]

[a] *Department of Applied and Molecular Ecology, Waite Campus, Adelaide University, Glen Osmond, SA 5064, Australia*
[b] *Plant Virology, CSIRO Division of Plant Industries, Waite Campus, Glen Osmond, SA 5064, Australia*
[c] *Department of Entomology, University of Georgia, Athens, GA, USA*
[d] *National Museum of Kenya, P.O. Box 40658, Nairobi, Kenya*

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Abstract

The ichneumonid endoparasitoid *Venturia canescens* successfully develops inside the hemocoel of another insect by using maternal protein secretions, including nucleic acid-free virus-like particles (VLPs), to manipulate host physiology. These VLPs consist of four major proteins, which are produced mainly in the calyx tissue and transferred into the host insect together with the egg. One of the protein-coding genes (*vlp1*), with similarities to phospholipid-hydroperoxide glutathione peroxidases (PHGPx), exists in allelic forms producing two protein variants with different protein properties. Here, we summarise observations indicating that oocytes and eggs are the source of reactive electrons, which potentially damage the lining and membranes of calyx tissues. We discuss the possible role of VLP1 in counteracting the damaging effects of oxidised phospholipids on membranes surrounding VLPs in the calyx lumen.

Keywords: Reproductive success; Egg maturation; Superparasitism; Phenoloxidase-like activity; Reactive oxygen; Phospholipid-hydroperoxide glutathione peroxidases

1. Introduction

Endoparasitoid insects deposit their eggs inside the body of another insect, exposing the larval stages of the developing parasitoid to the potentially fatal influence of the host's immune system. To facilitate the development of their young, many female endoparasitoids produce a range of ovarian and venom gland secretions that are injected into the host during oviposition (Beckage, 1997; Godfray, 1994; Salt, 1973), where they interfere with the defense system and other host functions (Salt, 1970; Schmidt et al., 2001). In the case of the solitary endoparasitoid wasp *Venturia canescens* these secretions include nucleic acid-free virus-like particles (VLPs) that are produced in calyx cells of the reproductive tract (Bedwin, 1979; Rotheram, 1973). The particles comprise four major proteins of 60, 52, 40 and 35 kDa in size (Feddersen et al., 1986), which are assembled inside the nuclei of calyx cells and surrounded by a proteaceous unit membrane, like those of morphologically similar DNA-containing ichnoviruses (Norton et al., 1975). The particles are released from the cells by a budding process (Stoltz and Vinson, 1979). Particles in the cytoplasm of calyx cells enter microvilli located on cell membranes facing the lumen and become separated from the cell by the formation of membrane-vesicles containing the protein particle. The calyx-derived membrane surrounding the coated particle is clearly visible during the budding process, but becomes less obvious after separation from the calyx cell (Feddersen, 1982; Rotheram, 1973). The acquisition of a membrane during budding and its disappearance inside the calyx lumen has also been described for ichnoviruses (Norton et al., 1975; Stoltz

* Corresponding author. Tel.: +61-8-8303-7252; fax: +61-8-8303-7109.
E-mail address: otto.schmidt@adelaide.edu.au (O. Schmidt).

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and Vinson, 1979), but nothing is known about its functional significance.

Cloning of *V. canescens* VLP genes revealed a gene (*vlp1*) coding for the 40 kDa protein with similarities to vertebrate phospholipid-hydroperoxide glutathione peroxidases (PHGPx) (Hellers et al., 1996), and a second gene (*vlp2*) that coded for the related 60 and 52 kDa proteins as cytoplasmic proteins (Reineke et al., 2002). The latter are of unknown function but may be important for the structural assembly of the VLP. Further studies of the VLP1 protein indicated that despite extensive similarities to PHGPxs from other organisms, a crucial cysteine residue at the active site was missing in the protein sequence (Hellers et al., 1996) and that both native and recombinant VLP1 had no measurable enzyme activity (Li et al., 2003a). However, selenium-containing enzymes, such as PHGPx, may be unable to function in the calyx lumen, since enzyme activity is dependent on continuous supply of reduced glutathione, which is probably absent in the calyx lumen. Given the large amounts of VLP1 protein found in the particle, and its relatively high degree of protein similarity to functional PHGPx proteins, this suggests the VLP1 function may involve recognition of, and binding to modified lipids such as phospholipid-hydroperoxides, in a non-enzymatic one-to-one reaction.

The two allelic genes differ in the deletion of a 54 bp tandem repeat sequence in the coding region, producing two allelic VLP1 proteins (Hellers et al., 1996) differing by an internal deletion of 18 amino acids (named "repeat plus", RP and "repeat minus", RM). Since the repeat sequence is between the PHGPx domain and a lipophilic N-terminal domain (Beck et al., 1999; Hellers et al., 1996), the allelic VLP1 proteins may have different tertiary structures and therefore differ in their functional properties. The VLP1 protein is processed inside the host, *Ephestia kuehniella*, by an Arg-specific trypsin-like protease, generating an 18.5 kDa peptide comprising the PHGPx domain (Beck et al., 2000), which is the same in both alleles. This suggests that any phenotypic differences due directly to the VLP1 allelic forms will be observable in the reproductive tissues, where the VLP1 exists as an unprocessed precursor, while other phenotypic differences may be pleiotropic effects or other unrelated genetic differences.

When asexual wasps homozygous for one of the two alleles were examined, a number of phenotypic differences were observed. The calyx glands of RM-wasps were larger than those of RP-wasps, with extended VLP-filled membrane systems, and the total amount of secreted VLP1 protein in the calyx was less for the RM-wasps than the RP-wasps. There were differences in the transfer of eggs through the calyx to the oviduct, with 7-day-old RM-wasps having significantly more eggs in the calyx and significantly fewer in the oviduct, compared to RP-wasps (Beck et al., 1999). Analysis also revealed complex differences in the reproductive success of the two strains under intra- and inter-strain superparasitism (Beck et al., 2001; Li et al., 2003b).

An examination of field-collected asexual wasps from two separate locations, as well as homozygous wasps from a mixed interbreeding sexual culture revealed that the three RM-cultures all showed similar, but less pronounced, patterns of morphological and functional characters to the laboratory RM-line, while the patterns for the three RP-cultures were similar to the laboratory RP-line (Li et al., 2003b), consistent with the suggestion that the VLP1 protein is involved in the phenotype and the functional differences are pleiotropic effects of the VLP1. The reduced phenotype in all three RM-cultures also suggests that, in the case of the laboratory culture, at least one other gene may be involved, playing either an additive or enhancing role.

Subsequent investigation of asexual RM- and RP-females from a culture newly established from field-collected wasps (Amat et al., 2003), reported that the calyx egg load phenotype expressed by the original laboratory colony RM-line was observed in both RM and RP strains, but again, in a less pronounced form. While this confirms that wasps displaying both the RM- and RP-phenotype can persist under field conditions, the occurrence of an RM-phenotype in an RP-genetic line is on the surface a puzzle. One possibility is that the observed association between the RM-VLP1 and the RM-phenotype for the four lines (Beck et al., 1999; Li et al., 2003b) is simply a coincidence. Alternatively, consistent with Li et al. (2003b), the laboratory RM-phenotype may be the result of more than one gene acting additively, but able to act separately. Given several studies on the laboratory cultures demonstrating that the RM-phenotype has a relative competitive advantage under conditions that favour superparasitism (Beck et al., 1999; Reineke et al., 2004), is probably not surprising that strains displaying different pathways to the RM-phenotype occur in field populations.

Considering that the observed differences in the RM-phenotype are not fully penetrant (Beck et al., 1999; Li et al., 2003b), and indications that the phenotype is dependant on culture conditions (Roberts, unpublished data), suggests the RM-phenotype may also involve a non-genetic determinant. One possibility is the action of a pathogen (Amat et al., 2003). Candidates known to be associated with reproductive tissues include viruses (Reineke and Asgari, this issue) and the bacteria *Wolbachia* (West et al., 1998). However, as the RM-phenotype was observed in sexual RM-females but not in sexual RP-females derived from an interbreeding culture, it is difficult to see how the RM-phenotype could be due exclusively to the action of a pathogen. Alternatively, the variability may be due to phenotypic plasticity. Maternal transmission of an inducible

character state has recently been reported (Rahman et al., 2004), where tolerance to a *Bacillus thuringiensis* formulation in *E. kuehniella* correlated with an elevated immune response, which could be induced by pre-exposure to a low dose of the formulation and, once induced, transmitted to following generations through the female line.

To explore the physiological basis of the RM-phenotype, and the function and hence possible role of the VLP1 protein in generating that phenotype, we examined the reproductive tracts of laboratory colony RM- and RP-females. In particular, given the high degree of sequence homology between the VLP1 and PHGPx, we investigated processes that could lead to the production of oxidised phospholipids. Here, we report a phenoloxidase (PO)-like activity in oocytes and eggs that may be responsible for the leakage of reactive electrons with the potential to damage biological substances, such as calyx-cell and VLP membranes. The PO-like activity is strongest in the basal section of the ovarioles and the apical half of the calyx region, is more pronounced in the reproductive tracts of RM-females, and corresponds to the area of the calyx that in RM-females shows extensive membrane damage and reduced expression of the VLP1-protein.

2. Methods

2.1. Insect colonies

The initial cultures of wasps named RP and RM were clonal RP- and RM-lines of *V. canescens* as described in (Beck et al., 1999). Hosts were *E. kuehniella*, reared on a 10:2:1 mixture of oat bran, wheat germ, and dried brewers yeast. All experiments were conducted and cultures maintained at 25 ± 1 °C, under a constant light–dark regime (L14:D10).

Wasps were reared in cylindrical clear plastic tubs; height 20 cm and diameter 15 cm. Four or five adult wasps were placed into each container with 40–50 hosts until the wasps died (around 24–36 h). Upon emergence the wasps were removed from the culture and kept in groups of 10 or less in gauze-covered 425 ml clear plastic cups (Party Rite Jumbo Tumblers®, Harris Paper Pty. Ltd., West Heidelberg, Australia) with a 50% honey solution.

2.2. Ovary staining

Whole ovaries were dissected from wasp abdomen and fixed overnight in phosphate buffered saline (PBS) containing 4% paraformaldheyde (PFA) and 1% Triton X100. The ovaries were washed four times in PBS over a period of several hours before incubation in anti-VLP antiserum (Feddersen et al., 1986), anti-PO antiserum (a gift from M. Kanost), or corresponding pre-sera in a dilution of 1:500 in PBS for 8 h. The ovaries were then washed four times in PBS with one wash overnight. TRITC-conjugated secondary antibodies (goat anti-rabbit IgG, Sigma) diluted 1:50 in PBS were applied for 8 h, followed by one wash overnight in PBS. The ovaries were then mounted in anti-fade solution (2% (v/v) 1,4-diazabicyclo-(2.2.2)-octane in 1 M Tris–HCl pH 7.5 made up in 80% glycerol) and inspected under indirect UV-light using confocal microscopy.

2.3. Tissue sections

Frozen tissue sections of ovaries were performed as described (Dequin et al., 1984). Briefly, ovaries were immersed in optimal cutting temperature (OCT) tissue compound Tec (Miles Scientific) and frozen in liquid nitrogen and sectioned at 10 μm at −20 °C using a cryocut microtome (Reichert-Jung). Sections were placed on subbed (0.02% Chrom Alum in 0.2% gelatin) slides and fixed in 4% PFA in PBS for 20 min, washed three times in PBS and dehydrated by incubating in an ethanol gradient (30–100% in five steps). Sections were then incubated in anti-VLP1 antibodies (Hellers et al., 1996), anti-VLP antibodies (Feddersen et al., 1986) and the corresponding pre-sera. After three washes, sections were incubated with FITC-conjugated secondary antibodies (goat anti-rabbit IgG, Sigma) and mounted in anti-fade for inspection under indirect UV-light. Sections from more than 10 animals were analysed.

2.4. DOPA-staining

PO-like activity can be detected after incubation with 20 mM DL-DOPA (close to saturation in PBS, pH 6.8), which shows blackening of oocytes at late stages of oogenesis and eggs inside the calyx and oviduct. The differential staining of ovarian tissues is reproducible, while the intensity of staining depends on the DL-DOPA concentration and time of reaction. The staining can be inhibited by phenylthiourea (PTU) and tropolone, which are chelating substances that inhibit copper-containing enzymes, such as PO (Li et al., 1996). The activity is not inhibited by protease inhibitors that prevent hemolymph PO-activity, such as calyx fluid-specific serpin (Beck, 1998) and p-AMSF, an indication that the PO-like enzyme is already activated at the time of dissection. Since oocytes and eggs do not show obvious signs of darkening in the absence of DL-DOPA, it is likely that DL-DOPA is not the natural substrate for this enzyme and that the enzyme activity may have different functions, such as immune functions or cross-linking of chorion proteins.

To identify PO-like enzymes "in-gel" staining of ovarian tissue and hemolymph extracts was performed as described (Xylander and Bogusch, 1992). Protein extracts were dissolved in non-reducing buffer (0.5% SDS, 10% glycerol, 0.2% Bromophenol blue in 1 M Tris–HCl pH 6.8) without boiling. Fifty microgram protein per sample were loaded and after electrophoretic separation gels were washed for 60 min in PBS containing 2% Triton X-100 and then washed twice for 5 min in PBS. Gels were stained in 20 mM DL-DOPA solutions until a black band of melanised substrate appeared and rinsed thoroughly in water.

2.5. NBT staining

The presence of reactive electrons in the ovary was determined by incubating live ovaries in a solution of 20 mM NBT, which is reduced by reactive electrons to form a formazan that precipitates as a blue dye at the site of reduction. As with melanisation, the differential staining of ovarian tissues is reproducible, while the intensity of staining depends on the NBT concentration and time of reaction. Since reactive electrons are usually (Nappi et al., 1995), but not always (Fridovich, 1995), associated with reactive oxygen radicals (ROS), the NBT-staining can be used in a first approximation as an indication for potential lipid peroxidation.

3. Results

3.1. Oxidative reactions in the V. canescens ovary

When dissected ovaries are incubated with DL-DOPA, oocytes and eggs turn black (Fig. 1A, showing a dark-field picture, where melanised black dots appear white) in a reaction that is similar to melanisation (Fig. 3B,C). The differential staining is an indication for an enzyme-like activity. When ovaries were stained with anti-PO antibodies, the staining coincided with the DL-DOPA-staining and is particularly strong in areas where ovarioles connect to the calyx (Fig. 1B). In-gel staining of ovarian tissues confirmed a band of enzyme activity after electrophoretic separation of ovary extracts, which is different from hemolymph PO, which is not detectable under these conditions (Fig. 2). A PO-like activity has been reported in mosquito eggs, where eggs turn black in the presence of oxygen (Li et al., 1996). Since *V. canescens* eggs show no signs of darkening reactions in the absence of DL-DOPA, even under extended periods in oxygen-rich environments, the observed melanization is probably not the natural reaction and the PO-like activity is more likely involved in other functions, such as other immune functions or cross-linking of egg-shell proteins.

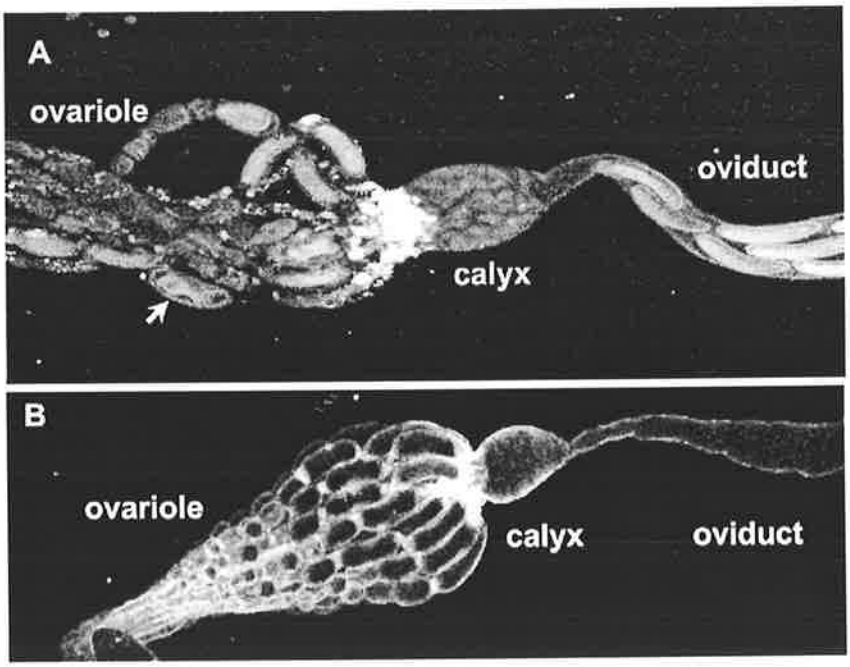


Fig. 1. Oxidising activities in an RP-ovary of *V. canescens*. (A) Whole ovary and oviduct incubated with DL-DOPA and inspected in dark field showing black melanisation as white reflections. Note the strong reactions at the site of transition between ovarioles and entry of eggs into the calyx tissue; egg-shell and ooplasm are stained (arrow). (B) Localisation of a PO-like protein in ovaries using anti-PO antibodies, TRITC-conjugated secondary antibodies and inspection using confocal microscopy in the TRITC-channel. No significant staining was observed with the preserum or secondary antibodies alone (not shown).

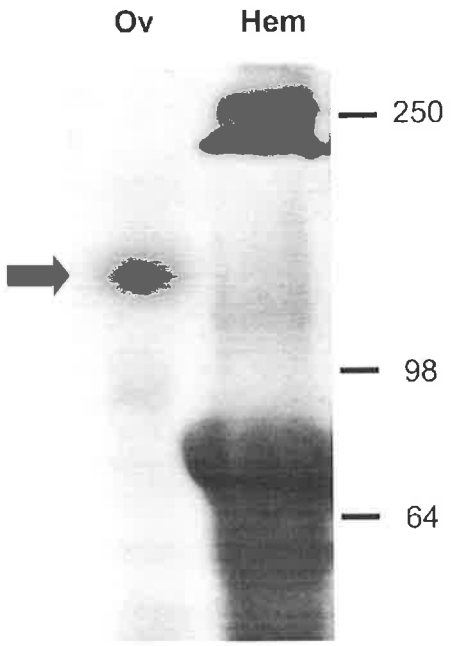


Fig. 2. In-gel staining of ovarian and hemocyte extracts. Separation of protein extracts from ovarian tissues (Ov) and hemocytes (Hem) under non-reducing conditions. After electrophoretic separation on a 7.5% SDS-PAGE the gel was stained with DOPA and subsequently with Coomassie blue.

Whatever the function of a PO-like activity, the copper-ion can potentially create damaging reactive electrons. We therefore incubated ovaries with NBT, which is diagnostic for reactive electrons (Fridovich, 1995). The blue staining was localised to vitellogenic oocytes and eggs passing through the calyx tissue and stored inside the oviduct (Fig. 3A). When NBT staining was examined in calyx tissue, it was apparent that the staining is reduced in areas where VLPs are found (Fig. 4). This suggests that eggs of the parasitoid *V. canescens* generate reactive electrons that are potentially damaging to ovarian tissues and that calyx protein secretions may counter-act some of the damaging effects.

3.2. Damage to calyx tissue

If eggs are a source of damage to surrounding tissues, the question is what is the phenotypic manifestation and how does the wasp cope with the damage? To answer the first question, we examined VLP-expression on frozen tissue sections of ovaries to identify any possible damage due to the passage of eggs from the ovary to the oviduct. When sections of ovaries from newly emerged wasps were analysed no significant damage was detected, whereas in some older RM-wasps tissue damage was observed in calyx tissues as autofluorescent bright spots, which indicate oxidative reaction products in damaged cells (Fig. 5). Moreover, expression of VLP proteins was diminished in calyx tissue-areas facing the entry of eggs on their passage from ovarioles into the oviduct. No obvious damage was noticeable to the oviduct.

3.3. Phenotypic changes in allelic ovaries

Since VLP1 with conserved PHGPx protein sequences may recognise and bind modified phospholipids,

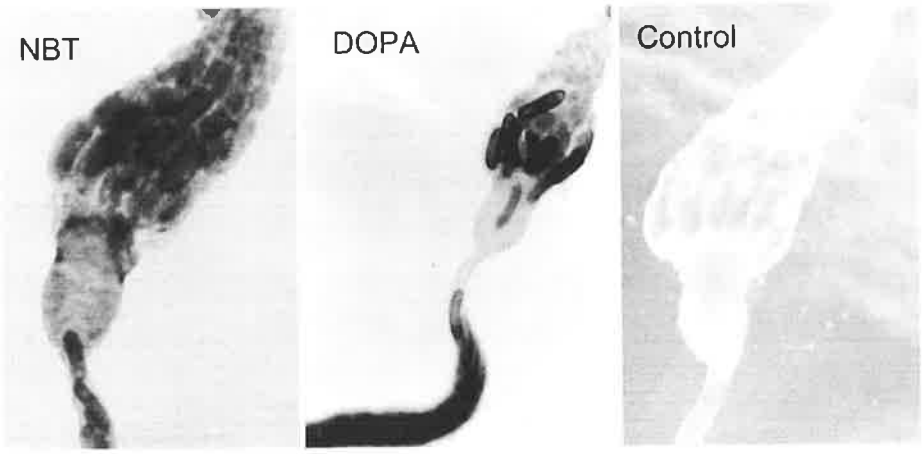


Fig. 3. Live ovaries stained with NBT and DL-DOPA. (A) Ovaries stained with a PBS solution containing 20 mM NBT. The yellow NBT is reduced by reactive electrons to form a formazan that precipitates as a blue dye (shown in black) at the site of reduction. Since reactive electrons are usually (Nappi et al., 1995), but not always (Fridovich, 1995), associated with reactive oxygen radicals (ROS), the NBT-staining can be used in a first approximation as an indication for potential lipid peroxidation. Staining in vitellogenic parts of the ovarioles is mostly localised to other structures in addition to oocytes, such as nurse cells, whereas in later stages the oocytes are predominantly stained; (B) Ovaries stained with a PBS solution containing 20 mM DL-DOPA. Since oocytes and eggs do not show any signs of darkening it is likely that DL-DOPA is not the natural substrate for this enzyme and that the PO-like activity may have different functions, such as other immune functions or cross-linking of egg-shell proteins; (C) Untreated ovaries.

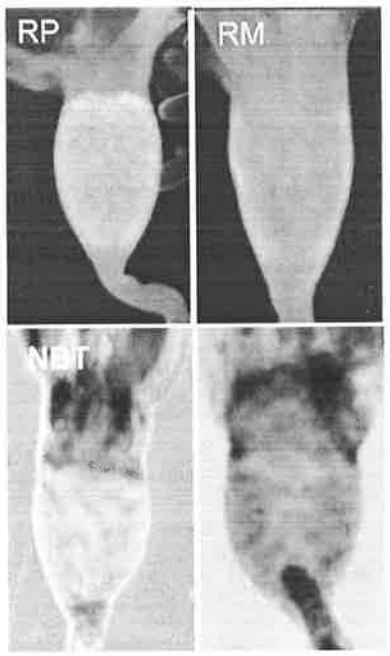


Fig. 4. Localisation of VLPs and NBT staining. (Above) Whole ovaries displaying RP (left) and RM (right) phenotypes were incubated in anti-VLP-antibodies and bound antibodies visualised with FITC-conjugated secondary antibodies and inspected under indirect UV-light. (Below) Ovaries with RP (left) and RM (right) phenotypes stained with a PBS solution containing 20 mM NBT.

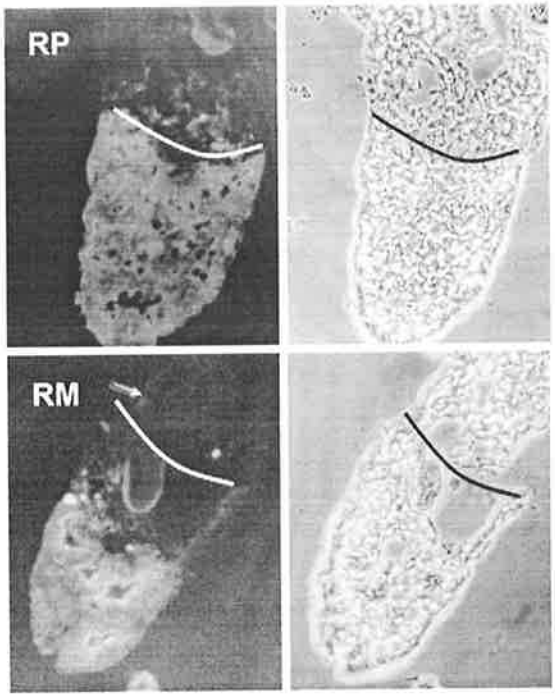
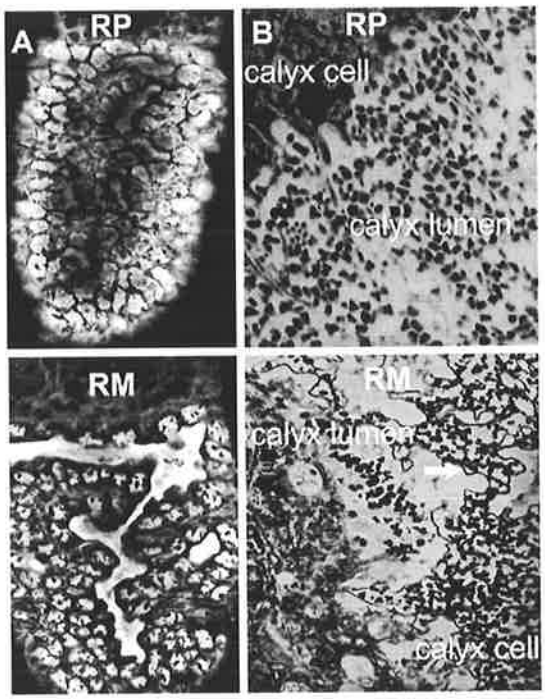


Fig. 5. Frozen section of calyx tissue from wasps displaying RP- and RM-phenotypes stained with anti-VLP1 antibodies, visualised with FITC-conjugated secondary antibodies and inspected under indirect UV-light. Phase contrast pictures of the same section are shown on the right. Sections representing RP- and RM-phenotypes are shown. Similar results were obtained with anti-VLP antibodies (not shown). The morphologically visible separation of calyx tissue and ovarioles are highlighted with a black line. Note that the expression of VLP proteins was diminished in parts of calyx tissues facing the entry of eggs on their passage from ovarioles into the oviduct. This area also shows morphological signs of cell and tissue damage, such as yellow autofluorescent dots inside damaged cells (arrow), which is an indication for necrosis.

thereby altering the VLP-membrane structure (Li et al., 2003a), we investigated possible effects of VLP1 on the surrounding membrane. Calyx tissues from the two allelic strains were examined for morphological differences. Using confocal and electron microscopy the most dramatic difference between the two tissues was the presence of additional membranes in the lumen of RM-calyx tissues (Beck et al., 2001), which probably derive from membranes surrounding VLPs, increasing in size by the merging of membranes containing VLPs with the age of the wasp (Fig. 6). The fact that these additional membranes in the calyx lumen are rarely observed in RP-wasps suggests that the allelic difference in the VLP1 protein may be responsible for the phenotype.

4. Discussion

We observed that oocytes and eggs are a source of reactive electrons, consistent with the existence of copper-containing enzymes that are active throughout oogenesis and egg storage in the oviduct. Although the exact identity and function of the enzyme remains to be determined, copper-containing proteins have the potential to produce reactive electrons that can leak from oocytes passing from ovarioles into the oviduct with damaging effects on calyx tissues. The activity is found in nurse cells, the ooplasm and on the egg surface, probably in the egg-shell. Other copper-containing proteins, such as hemocyanin, are not ruled out, but have not been found in higher insects (Hagner-Holler et al., 2004; Sanchez et al., 1998). PO-like enzymes have been reported in mosquitoes, where the enzyme is involved in tanning of the egg-shell (Li, 1994). However, eggs dissected from ovaries or parasitised caterpillars do not show obvious signs of darkening, which suggest that melanization is not the natural reaction and that the PO-like activity in *V. canescens* may have different functions. Since PO-like enzymes are involved in the cross-linking of cuticle proteins a similar function may be possible for egg-shell proteins. Cross-linking of chorion proteins in Diptera involve peroxidases (Keramaris et al., 1996; Margaritis, 1985), but may include PO-like activities (Li et al., 1996; Nappi et al., 1995). In most insects, these enzymes are inactivated at the end of oogenesis



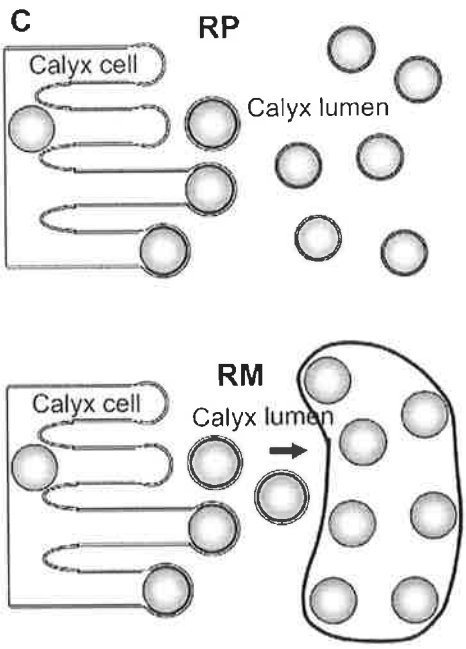


Fig. 6. RM-specific alterations in the calyx tissues. (A) RP and RM ovaries stained with anti-VLP1 antibodies and inspected under confocal microscopy inside the calyx lumen as shown previously (Beck et al., 2001). Note the large strongly stained membrane-surrounded areas in the lumen of RM-tissue. The large nuclei, where VLPs are assembled are less stained in RM- compared to RP-tissues; (B) Electron microscopic images of calyx lumen and surrounding tissues from RP- and RM-females as shown previously (Beck et al., 2001). Note the electron-dense membrane-staining around VLPs in the calyx lumen of RM-wasps; (C) Possible events leading to RM- and RP-phenotypes in calyx lumen of *V. canescens*. VLPs emerging from calyx cell-derived microvilli have visible membrane bilayers. Given that membranes are exposed to reactive electrons leaking from eggs, the membrane lipids may be damaged by oxidation processes, leading to modified lipids. Phospolipid-hydroperoxides are recognised by the underlying VLP1, which binds the membranes to the protein particle (RP). In insects with altered VLP1 proteins (RM), the attachment may not be as effective and damaged membranes are prone to merge with each other forming large vesicles containing membrane-less particles. The presence of multiple eggs inside the calyx lumen of RM-wasps compounds the problems of reactive electrons leaking from the eggs with the result that calyx tissues become damaged and produce less calyx proteins.

with the completion of the chorion, when the enzyme is increasingly cross-linked to other chorion proteins. However, in hydropic eggs of some parasitoid species, the chorion hardening may be interrupted to allow additional extension of egg size after egg deposition (Quicke, 1997). Although it is not known how this intermission of cross-linking is regulated, the fact that the PO-like activity is detected in *V. canescens* long after the oocyte has left the ovariole suggests that eggshell hardening may not be completed during oogenesis to allow the adjustment of final egg size inside the host hemocoel.

If the nature of hydropic egg maturation involves incomplete inactivation of an oxidising enzyme, the leakage of reactive electrons during passage and storage of oocytes poses a threat to the surrounding tissues and cells. Reactive electrons, which are predominantly manifest in reactive oxygen species, such as hydroperoxides, are particularly damaging to lipids. In fact, PHGPx is unique in recognising phospholipid-hydroperoxides inside membrane bilayers and reducing oxidised phospholipid inside membranes (Schuckelt et al., 1991). The observed protein sequence similarity of VLP1 and vertebrate PHGPxs (Hellers et al., 1996), suggest that the ability to recognise and bind phospholipid-hydroperoxides in VLP-membranes may be retained (Li et al., 2003a). Taken together with our studies of two *V. canescens* lines with allelic VLP1 proteins, we propose a possible role of VLP1 in altering the membrane surrounding VLPs in the calyx lumen with dramatic morphological effects. This is also the first, albeit indirect, evidence that VLP proteins play a functional role in the female ovary by protecting the calyx tissue against the deleterious effects of an egg-derived oxidising influence.

What is the possible mode of action of VLP1 in the calyx lumen? Ichnoviruses (Norton et al., 1975) and VLPs (Schmidt and Schuchmann-Feddersen, 1989) budding from calyx cells via microvilli are surrounded by calyx cell-derived membranes. However, the structural features of membrane bilayers "disappear"

shortly after the particles are released into the calyx lumen (Stoltz and Vinson, 1979) by an unknown process. It is tempting to speculate that PHGPx-like proteins interact with membrane phospholipids thereby altering the lipid–protein composition after particles are released from the calyx cell and membranes are exposed to reactive electrons from eggs passing through the calyx tissue. Under exposure to an oxidising source, oxidised phospholipids are probably the first membrane components to be modified, accumulating in the VLP-membrane as phospholipid-hydroperoxides (Korytowski and Girotti, 1999). VLP1 proteins in the underlying particle may recognise these modified lipids and bind to them, but because enzymatic reduction of the lipid molecule is lacking, the linkage will be retained and the membrane will eventually become closely attached to the underlying protein particle. In the calyx of RM-females, the structurally different VLP1 variant may not be as effective in absorbing the membrane to the particle, leaving the damaged membrane exposed to merging with membranes from other particles, thereby creating large membrane systems containing VLPs (Fig. 6). These membrane systems are an obstacle to the passage of eggs from the ovarioles to the oviduct thereby generating the RM-calyx phenotype, where eggs accumulate inside the calyx lumen instead of being transported into the oviduct (Beck et al., 1999).

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We want to dedicate this paper to George Salt, who pioneered *Venturia* as a model system for parasitoid host relationships. His ideas still inspire and guide our approaches to this fascinating biological interaction. We thank two anonymous reviewers for constructive comments to the manuscript. This work was supported by ARC grants to O.S.

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Appendix 1B.

Induction and transmission of Bt-tolerance in the flour moth *Ephestia kuehniella*

Rahman MM, **Roberts HLS**, Sarjan M, Asgari S, Schmidt O (2004) *Proceedings of the National Academy of Science* **101**:2696-2699

Rahman, M. M., Roberts H. L. S., Sarjan, M., Asgari, S. & Schmidt, O. (2004). Induction and transmission of Bacillus thuringiensis tolerance in the flour moth Ephestia kuehniella. *Proceedings of the National Academy of Sciences of the United States of America, 101*(9), 2696-2699.

Appendix 1C.

The development of the endoparasitoid *Venturia canescens* in Bt-tolerant, immune induced larvae of the flour moth *Ephestia kuehniella*.

Rahman MM, **Roberts HLS**, Schmidt O (2004) *Journal of Invertebrate Pathology* **87**:129-131

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Short Communication

The development of the endoparasitoid *Venturia canescens* in Bt-tolerant, immune induced larvae of the flour moth *Ephestia kuehniella*

M. Mahbubur Rahman, Harry L.S. Roberts*, Otto Schmidt

Insect Molecular Biology Laboratory, School of Agriculture and Wine, University of Adelaide, Glen Osmond, SA 5064, Australia

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Abstract

We examined the survival and development of the endoparasitoid *Venturia canescens* in a Bt-tolerant laboratory strain of the flour moth *Ephestia kuehniella*, in which Bt-tolerance has been shown to be associated with an inducible elevation of the insect's immune response. The results indicate the elevated immune status associated with Bt-tolerance does not confer cross-protection against parasitism by *V. canescens*. No significant difference was observed in the rate of emergent wasps from parasitised Bt-tolerant and Bt-susceptible hosts. In addition, wasps from Bt-tolerant hosts had longer development times and were larger than wasps from Bt-susceptible hosts.

Keywords: Integrated pest management; *Venturia canescens*; *Ephestia kuehniella*; *Bacillus thuringiensis*; Biological control

1. Introduction

Parasitoids are effective natural enemies of many pest species and are widely used in conjunction with biopesticides such as *Bacillus thuringiensis* (Bt) in integrated pest management (IPM) programmes. Previous research has found little evidence of direct negative effects on parasitoids of Bt-formulations or of Bt-transgenic plants, nor of Bt-resistant hosts acquiring cross-resistance to parasitoids (Glare and O'Callaghan, 2000; Schuler et al., 2004), indicating that parasitoids can be employed in a complimentary fashion with the use of Bt-formulations. However, while in previous studies host Bt-resistance has typically been due to alterations in gut protease activity or receptor insensitivity, recent research has shown that tolerance to Bt endotoxins in a laboratory strain of the flour moth *Ephestia kuehniella* (Lepidoptera: Phycitidae), a common pest of stored food products, is associated with an inducible increase in the rate of the melanization reaction in haemolymph (Rahman et al., 2004), a hallmark of an elevated immune response in insects (Soderhall and Cerenius, 1998). Similar results have also been observed for the cotton bollworm *Helicoverpa armigera* (Ma et al., under review) and the diamondback moth *Plutella xylostella* (M. Sarjan, unpublished data), suggesting that the initial development of Bt-tolerance through immune-related processes may be a common phenomenon. Since the capacity of a host to overcome parasitization in large part depends on the effectiveness of its immune response, this raises the question of whether the immune induction associated with Bt-tolerance results in cross-protection against parasitism. To answer this question we examined the survival and development of the endoparasitoid *Venturia canescens* Grav. (Hymenoptera: Ichneumonidae) in Bt-tolerant *E. kuehniella*.

* Corresponding author. Fax: +61-83794095.
E-mail address: harry.roberts@adelaide.edu.au (H.L.S. Roberts).

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2. Materials and methods

The toxin used was a commercial formulation of Bt endotoxins (DelfinWG, Sandoz (now Syngenta), North Ryde, NSW), consisting of Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, proteins, and spores.

The initial Bt-susceptible colony of *E. kuehniella* was a long established laboratory colony that had been maintained without selection for over 100 generations. The Bt-tolerant *E. kuehniella* originated from the colony described in Rahman et al. (2004), and subsequently maintained on diet containing 4000 ppm of the Bt-formulation. Both Bt-tolerant and Bt-susceptible larvae used in the study were reared from egg-hatch on toxin-free diet. All hosts were maintained as per Rahman et al. (2004). The wasps were a clonal RP line of *V. canescens*, maintained as per Roberts and Schmidt (2004).

In the study, 150 Bt-susceptible line and 100 Bt-tolerant line larvae, ranging in mass from 10 to 50 mg, were each parasitised once by *V. canescens*. Following parasitism each host was weighed and then maintained individually in a glass vial until parasitoid emergence. Upon emergence parasitoids were killed by freezing, their head capsule width measured and development time recorded. To assess whether host line differentially affected wasp size to mass ratio a sample of 30 wasps emerging from each host line were dried in an oven at 50 °C for 24 h and then weighed. Procedures for obtaining single parasitised hosts and determining survivorship and developmental parameters followed the methods of Roberts et al. (2004). The immune status of eight samples *E. kuehniella* larvae from each line was determined by melanisation assays according to the method of Rahman et al. (2004).

Data were analysed using the general linear model (GLM) platform, JMP V4.0.4 (SAS, 2001), with continuous factors centred by their means (Neter et al., 1990). Analyses started with full models with all interactions, and non-significant interactions were dropped if $p > 0.25$ (Winer, 1971).

3. Results

Compared to Bt-susceptible larvae, cell-free haemolymph (Fig. 1) from Bt-tolerant larvae showed significantly increased melanization reactions ($F = 157.8$, df = 1,108, $p < 0.0001$), a hallmark of an elevated immune response in insects (Soderhall and Cerenius, 1998).

Neither host line nor mass had a significant effect on parasitism success (rate of emergent wasps from Bt-tolerant hosts = 87%, from Bt-susceptible hosts = 88%).

Analysis by GLM of wasp development time with host line and host mass as factors revealed a significant difference between the host lines ($F = 81.3$, df = 1,212, $p < 0.0001$; least squares mean development time: in Bt-tolerant hosts = 25.8 day, in Bt-susceptible hosts = 24.0 day; Fig. 2A) as well as a small but significant positive relationship between host mass and development time ($F = 9.41$, df = 1,212, $p = 0.002$). The interaction between host line and host mass was not significant, indicating that the difference in development time of wasps from the two host lines was independent of host mass.

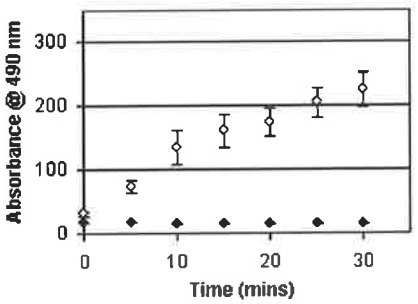

Fig. 1. Melanization assays of cell-free haemolymph from Bt-tolerant (open circle) and Bt-susceptible (closed circle) *E. kuehniella* larvae. Bars represent SEM.

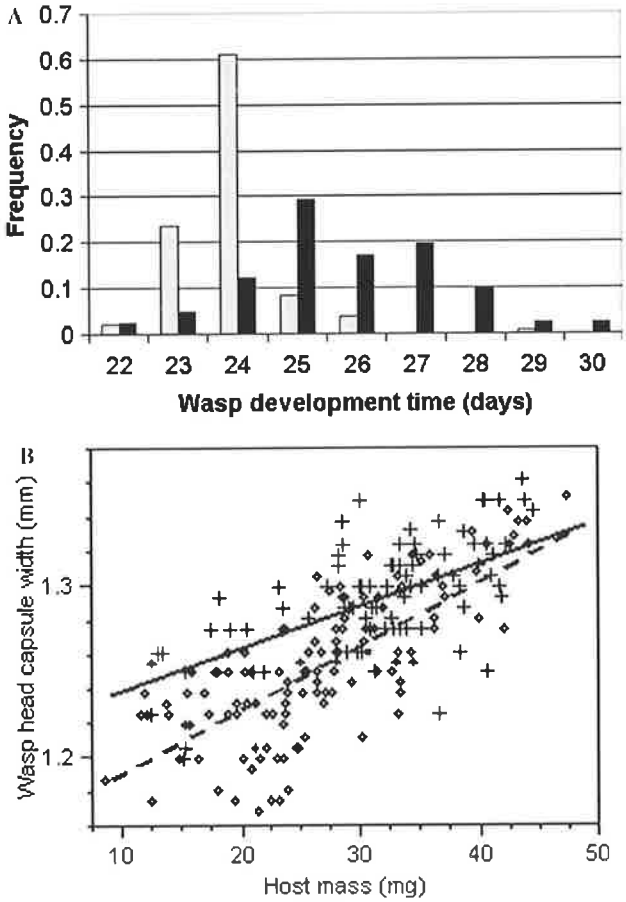

Fig. 2. Development of *V. canescens* in *E. kuehniella* larvae. (A) Histogram of development time in days of parasitoids in Bt-susceptible (grey bars) and Bt-tolerant (black bars) hosts. (B) The relationship between the mass of the host and the head capsule width of the emergent parasitoid from Bt-susceptible (diamond and dashed line) and Bt-tolerant (plus and solid line) hosts. Lines represent linear regression.

A similar analysis of wasp head capsule width revealed the effect of host line was significant ($F=40.8$, df $=1,211$, $p<0.0001$), with larger wasps developing in Bt-tolerant hosts. There was also a significant positive relationship between host mass and adult wasp head capsule width ($F=179.2$, df $=1,211$, $p<0.0001$), and a significant interaction between host mass and host line ($F=5.13$, df $=1,211$, $p=0.025$), indicative of the difference in adult wasp head capsule width between the two host lines becoming smaller as host mass increased (Fig. 2B).

Analysis by GLM of wasp head capsule width with wasp dry weight and host line as factors revealed the effects of host line were not significant, indicating that host line did not differentially affect the wasp head capsule width to mass ratio.

4. Discussion

The study found no significant difference in the rate of emergent wasps from the Bt-tolerant and Bt-susceptible lines of *E. kuehniella*, indicating that the elevated immune status associated with the Bt-tolerant line (Fig. 1) does not provide *E. kuehniella* with cross-protection against parasitism by *V. canescens*. The study also found that wasps from Bt-tolerant hosts had longer development times and were larger than wasps from susceptible hosts. The increase in wasp development time was independent of host mass, while the increase in wasp size (as measured by adult wasp head capsule width, Fig. 2B) was greatest for small hosts (which themselves have the greatest potential for growth) and smallest for large hosts (which have the smallest potential for growth). This suggests these effects represent a partial inhibition of embryonic or early instar parasitoid development in the Bt-tolerant hosts, such that the initial stage of parasitism, when the host is still able to feed and grow, is extended relative to Bt-susceptible hosts.

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Appendix 1D.

Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of tolerant *Helicoverpa armigera* larvae?

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For: Insect Biochemistry and Molecular Biology

Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of resistant *Helicoverpa armigera* larvae?

Gang Ma, Harry Roberts, Muhammad Sarjan[1], Nicki Featherstone[2], Jelle Lahnstein[2], Ray Akhurst[3] and Otto Schmidt*

Insect Molecular Biology, School of Agriculture and Wine, University of Adelaide, Glen Osmond, SA 5064, Australia
[1]Faculty of Agriculture, University of Mataram, Lombok, Indonesia
[2]ACPFG, School of Agriculture and Wine, University of Adelaide, Glen Osmond, SA 5064, Australia
[3]CSIRO, Entomology, Canberra, ACT 2000, Australia.

*corresponding author:
Tel: 0618 8303 7252
Fax: 0618 8303 7109
Email: otto.Schmidt@adelaide.edu.au

Abbreviations: Bt, *Bacillus thuringiensis*; BSA, bovine serum albumine; Cry, crystal toxin from *B. thuringiensis*; DBM, diamondback mothl; DOPA, 3,4-dihydroxyphenylalanine; DTT, DL-dithiotreitol, GalNAc, N-acetylgalactosamine; HPL, *Helix pomatia* lectin; PBS, phosphate-buffered saline; ORF, open reading frame; PNA, peanut agglutinin; PTU, phenylthiourea; rpHPLC, reverse phase high pressure liquid chromatography; TFA, trifluoroacetic acid.

The nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI Data Bank: The *H. armigera* hexamerin sequence accession number is AY661710.

Abstract

Bacillus thuringiensis endotoxins (Bt-toxins) are the most important biopesticides used in controlling insect pests and vectors of diseases. The emergence of widespread resistance to Bt in some insect species is a serious threat to agricultural production. Analysis of Bt-resistant and susceptible laboratory strains of *Helicoverpa armigera* revealed elevated immune responses involving increased melanization and the presence of a soluble toxin-binding glycoprotein in the hemolymph and gut lumen of the resistant strain. We propose a resistance mechanism against toxins based on a systemic immune-induction that can be transmitted to the next generation by a maternal effect.

1. Introduction

Insecticides from the soil bacterium *Bacillus thuringiensis (Bt)*, a natural pathogen of many invertebrates including insects, are economically and ecologically important biological control agents (Shelton et al., 2002). Due to its benign environmental properties and absence of any harmful effects on humans, the *Bt*-endotoxins are increasingly employed in pest management programs in favour of synthetic pesticides. Recently, bacterial endotoxin genes have been expressed in crop plants, where they provide protection against a number of major insect pests, such as cotton bollworms (*Helicoverpa zea* and *Helicoverpa armigera*).

Reports of the emergence of resistance in field populations of DBM have highlighted the inherent danger of the evolution of resistance against this powerful insecticide (Ferre and Van Rie, 2002). The most effective and best-characterised resistance mechanisms are based on receptor-inactivation at the midgut membrane, for example involving the aminopeptidase N (Darboux et al., 2002) and the cadherin-like (Gahan et al., 2001; Morin et al., 2003) gene families. Other mechanisms known to impair Bt-toxicity include alterations to proteolytic activity of midgut extracts affecting protoxin processing and maturation (Oppert, 1999), and increased rates of replacement of damaged cells at the gut lining by stem cells (Martinez-Ramirez et al., 1999).

We have recently reported evidence of an additional mechanism, where Bt-tolerance is associated with an elevated immune response (Rahman et al., 2004). When larvae from a susceptible laboratory strain of the flour moth *Ephestia kuehniella* were selected for survival on progressively increasing levels of a Bt-formulation for five generations the resulting Bt-tolerant strain displayed a constitutively elevated immune response.

Treating larvae of the susceptible strain of *E. kuehniella* with sublethal doses of the *Bt*-formulation led to an elevated melanization reaction in the hemolymph, which in turn was correlated with an increase in tolerance against the toxin at a later stage of larval development. As susceptible larvae were immune-induced and exposed to *Bt*-toxin in the same generation, this experiment excluded the selection of a pre-existing resistance allele as the cause of increased tolerance to the toxin in pre-treated larvae.

Reciprocal crosses of tolerant and susceptible insects revealed the transmission of both the immune induction and tolerance to the toxin from one generation to the next by a maternal effect. Offspring of tolerant females and susceptible males (TxS) were significantly more tolerant than offspring from susceptible females and tolerant males (SxT). A possible mechanism for this effect is the incorporation of an immune-elicitor into the oocyte by an immune-induced female. The elicitor can interact with embryonic tissues to induce the immune system of the neonate so that by the time the neonate starts feeding the insect is already induced, thus increasing the chances of surviving the toxin.

Bioassays showed that the SxT neonates were significantly more tolerant than the SxS neonates, indicating a genetic contribution to the variation in the immune response in addition to the maternal effect (Rahman et al., 2004). Further, the level of the immune response in the TxS larvae, as measured by the scale of melanization reactions, was variable between the offspring of different mating pairs, but directly correlated with the degree of Bt-tolerance. The fact that the level of the immune response and Bt-resistance in the TxS larvae occurred as a continuum suggests that the observed variation in the magnitude of the potential immune response is determined by more than one gene.

When females from the SxT cross were back-crossed with males from the tolerant strain, no immune-induction was observed in the offspring. This confirms that the elevated immune-status in the tolerant population was based on a transient immune induction, which is initiated in each generation by a maternal effect. However, when offspring from the backcross were immune-induced by a sub-lethal dose of the toxin, the observed melanization reaction was significantly greater than that detected in immune-induced susceptible larvae, confirming that the genetic disposition to respond to an elicitor was genetically determined by alleles that were different in the tolerant compared to the susceptible population (Rahman et al., 2004).

The molecular basis of the Bt-tolerance in this strain of *E. kuehniella* is not known. However the evidence that its magnitude is determined by more than one gene in turn suggests that it may involve multiple metabolic and regulatory pathways. For example, mutational changes in a gene product

involved in post-translational modifications, such as glycosyltransferases (Griffitts et al., 2001), may potentially interfere with pro-coagulant (Theopold et al., 2002), cell surface receptors (Oltean et al., 1999) and ovarian elicitor functions (Sen et al., 1998). If pro-coagulant molecules are transferred into the gut lumen as part of an immune response, it is possible that soluble immune components in the gut lumen interact with the mature toxin causing its inactivation by a coagulation or melanization reaction.

To examine whether immune-related Bt-tolerance mechanisms occur in other insects, we investigated differences between a susceptible and a Bt-resistant strain of the cotton bollworm *H. armigera*, an important insect pest (Akhurst et al., 2002; Akhurst et al., 2003). Mirroring the results for *E. kuehniella*, reciprocal crosses involving the susceptible and resistant strains revealed that tolerance involved a genetic and also a non-genetic component that was transmitted to the next generation by a maternal effect. Susceptible larvae fed with a diet containing a sub-lethal concentration of the toxin Cry1Ac subsequently displayed an elevated immune response in both the hemolymph and gut lumen. Further, the immune-induction was associated with an increased level of the larval serum protein hexamerin, which interacts with the toxin by an aggregation reaction to form an insoluble coagulum.

2. Material and Methods:

2.1. Bt-toxin

The Cry1Ac protoxin was purified from *B. thuringiensis* subsp. *kurstaki* HD73 by sucrose gradients (Liao et al., 2002). Protoxins were solubilized in a solution containing 30 mM Na_2CO_3 and 1% mercaptoethanol at pH 9.6 and digested with trypsin or gut juice extracts. Oligomeric Cry1Ac was obtained by performing digests at various time periods with *H. armigera* gut juice or trypsin (1ng/mg protoxin) and used after dialysis against PBS (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 6.8) buffer. Oligomeric toxin complexes were monitored on SDS-PAGE after extracting proteins from protoxin digests in electrophoresis loading buffer (2% SDS, 0.5mM DTT, TrisHCl, pH 8.5) at 65°C.

2.2. Insects

A laboratory strain of *H. armigera* was selected for resistance to Cry1Ac (Akhurst et al., 2002), and the resistant *H. armigera* insects were repeatedly crossed with susceptible insects resulting in nearly isogenic resistant (ISOC4) and susceptible (ANGR) strains (Akhurst et al., 2003). Resistant caterpillars were smaller and showed retarded growth and development on artificial diet, which at 25°C amounted to a two weeks delay in pupation of resistant compared to susceptible insects. Several variables, such as instar, size and age, were used to compare similar stage resistant and susceptible caterpillars. Alternatively, the two strains were kept at different temperatures to partly compensate for the differences in growth and development. All larvae were reared on artificial diet under a 14:10 L:D schedule (14).

2.3. Electrophoretic techniques

SDS polyacrylamide-gel electrophoresis on a Mini-Protean II electrophoresis unit (Bio Rad) was essentially performed according to (Laemmli, 1970). Molecular weights were determined using prestained SeeBlue^{TM} (Invitrogen) molecular weight markers. The proteins were blotted onto a nitrocellulose membrane (Amersham) as described by (Theopold et al., 1996). The amount of protein loaded was ca. 5 µg/lane in Western blots or as indicated in the figure legends. The blotting efficiency was determined by staining the blot with

Ponceau S. for Western-blots, peroxidase-conjugated PNA was used at a concentration of 1 µg/ml.

2.4. Immune-induction

The induction of melanization and other defence reactions was carried out by exposing 4^{th} or 5^{th} instar larvae from the susceptible strain to a sublethal dose of Cry 1Ac (1 µg per ml of artificial diet) for various time periods as indicated. Note that the same dose is used as a lethal dose for neonates (see below).

2.5.Melanization assays

Plasma from one or five caterpillar(s) was collected in the absence of PTU and light absorbance measured at a wavelength of 490 nm. Gut extracts were obtained by homogenizing gut, including gut content, in 1 ml PBS. Debris was removed by centrifugation (5,000*g*, 5 min) and the light absorbance (at 490 nm) of the supernatant was measured in the presence of 10 mM 3,4-dihydroxyphenylalanine (DOPA).

2.6. Coagulation assays

To detect coagulation-related aggregate formation, hemolymph from a single 5^{th} instar caterpillar was collected in 1.5 ml ice-cold PBS containing phenylthiourea (PTU) and hemocytes were removed by centrifugation (5,000*g*, 5 min at 6°C). The resulting plasma was divided into aliquots of 250µl to which lectins (0.1 µg each) or gut juice-digested Cry1Ac (between 0.1µg and 2µg) were added and mixed gently. After 5 min at room temperature the mixture was centrifuged for 5 min at 13 000*g*. Both pellet and supernatant were transferred into loading buffer and analysed by SDS-PAGE. In separate experiments gut juice extracts (0.1µg) or extracts from a bacterial strain lacking the toxin (0.1µg) were added to plasma without changing the composition significantly.

2.7. In-gel digestion of p85 protein

The protein gel bands of interest were excised and Coomassie blue stain removed by washing with 0.1 M NH_4HCO_3 and 50 % CH_3CN. Cystine bridges were reduced with β-mercaptoethanol and free cystine residues alkylated using iodoacetamide. The lyophilised gel piece was rehydrated by adding 200 µl of the digestion buffer containing 0.5 µg of trypsin (Sigma) and incubated for 16 h at 37°C. The digestion buffer was carefully removed, 200 µl of 1 % TFA was added to the tube containing the gel piece and the peptides were extracted.

2.8. Reverse Phase-HPLC

After digestion, the total reaction mixture was loaded onto a VYDAC® reverse phase C18 column and eluted at the flow rate of 0.2 ml/min using a gradient of 5 to 100% of buffer B (0.04% trifluoroacetic acid (TFA) in 70% acetonitril) against buffer A (0.05% TFA in water) over 78 minutes. Collected fractions were detected by absorbance at 214 nm and protein fractions were collected manually.

2.9. Peptide sequencing

Three peptide peaks from the rpHPLC (approximately 100 picomol) were vacuum-dried, reconstituted in 8M urea, containing 0.1M NH_4HCO_3 and 4mM DL-dithiotreitol (DTT) and finally alkylated by addition of sodium iodoacetate to a final concentration of 10mM. The samples were acidified with TFA to stop the reaction. Peptide sequencing was carried out using a Hewlet Packard G1000A Protein Sequencer.

2.10. Cloning of the p85-coding DNA

A degenerate primer HaAngr26F (GACGARCCNTTYGGNTTC) was designed from peptide No. 26 and used in conjunction with oligo-dT primers to amplify coding DNA sequences from RNA prepared from fat body of Bt-induced *Helicoverpa armigera* larvae.

A 400 bp cDNA fragment was obtained from the first RT-PCR, which had sequence homologies to *Heliothis* hexamerins (NCBI, blast search). Since 5'-RACEs only yielded short fragments, we used degenerate RT-PCR from the highly conserved regions of the hexamerin DNA sequence (located in the regions of the hemocyanin-M domain and the N-terminus), which gave a full-length cDNA fragment of 2442 bp and an ORF coding for 706 amino acids. All three sequenced peptides were contained in the protein; numbers 14 and 26 were in the C-terminus, whereas number 37 was near the N-terminus.

2.11. Genetic crosses

Crossing resistant and susceptible individuals will produce two different outcomes depending on the mode of transmission of the phenotypic trait. If resistance is caused by a recessive mutation expressed in developing offspring, F1 neonates will be susceptible. Transmission by a maternal effect is expected to produce resistant offspring from resistant females, while those from resistant males will be susceptible. The two scenarios are depicted

below to demonstrate the phenotypic differences of the two possible outcomes. In a putative sex-linked transmission the incidences of resistance in F1 offspring (in bold) are expected in opposite crosses and restricted to females, since females are hemizygous in lepidopteran species.

If resistance is due to a recessive trait, resistance is only detected if the gene is sex-linked (female offspring in cross shown on the right):

$\mathbf{X^rY}_F \times X^sX^s_M$ $\qquad$ $X^sY_F \times \mathbf{X^rX^r}_M$

F1: $X^rX^r_M$; X^sY_F $\qquad$ F1: $X^sX^r_M$; $\mathbf{X^rY}_F$

If resistance is based on maternal transmission, all offspring of resistant females (cross shown on the left) will be resistant. Similar outcomes are expected whether transmission occurs by maternal inheritance (e.g. mitochondrial gene mutation) or maternal imprinting.

$R(\mathbf{XY})_F \times S(XX)_M$ $\qquad$ $S(XY)_F \times R(\mathbf{XX})_M$

F1: $R(\mathbf{XX})_F$; $R(\mathbf{XY})_M$ $\qquad$ F1: $S(XX)_F$; $S(XY)_M$

Four crosses were performed, susceptible X susceptible (SxS), resistant X resistant (RxR), susceptible female X resistant male (SxR) and resistant female X susceptible male (RxS). For each of the crosses, similar weight, temperature-adjusted four-day-old larvae from 20 pairs of adults were exposed to a spore/Cry1Ac preparation of HD73 incorporated into the *H. armigera* diet, at a concentration of 1µg/ml as determined by in-gel comparison with BSA standard solution. Numbers of larvae used were: SxS n=144, RxR n=144, SxR n=192, RxS n=120. Mortality was recorded daily for seven days, at which point all susceptible insects had died. The data were analysed by Cox's proportional hazard model (Cox, 1970), using Efron's (Efron, 1977) partial likelihood method. To determine the differences between the sexes, the SxS cross was treated as the baseline hazard and the resistant males and resistant females as separate covariates, assumed to be acting multiplicatively. To determine the differences between the crosses, the SxS cross was treated as the baseline hazard and the other three crosses as separate covariates.

3. **Results**

3.1. Melanization in the hemolymph and gut lumen

To examine a possible association between Bt-resistance and an elevated immune status in the resistant strain, we measured the melanization reaction in cell-free hemolymph (plasma) as a first approximation of an induction of the humoral immune system (Shelby et al., 2000; Soderhall and Cerenius, 1998). When plasma of larvae from the resistant strain was analysed, a strong melanization reaction was observed within one hour whereas no significant melanization was observed in plasma from susceptible larvae (Fig. 1a). When gut extracts from resistant and susceptible larvae were examined in the presence of DOPA, higher melanization rates were observed in the gut extracts from resistant compared to susceptible larvae (Fig. 1b). Further, in gut wholemount preparations from resistant strains, the peritrophic membrane and the gut content were frequently darker, and in some extreme cases showed large black patches of melanization products, whereas the susceptible gut was always transparent and white (Fig. 2).

3.2. p85 is immune-induced

To examine whether immune-induction causes visible changes in the hemolymph, we compared the protein patterns of plasma from resistant and susceptible larvae. In general, for resistant larvae most of the major plasma proteins were significantly reduced in amounts relative to the susceptible larvae, even when plasma was collected on ice and dissolved in anti-coagulant buffer (not shown). Together with the observed differences in melanization reactions (Fig. 1a, Fig. 2), this suggested an increased coagulation reaction in the plasma of resistant larvae.

In plasma from non-induced susceptible larvae, several minor proteins in the range of 85-90 kDa that bind to Bt-toxin (not shown) and GalNAc-binding lectins were observed (Fig. 3). One of them has antigenic similarity to apolipophorin I (G. Ma, unpubl. data), and could be a proteolytic digestion product of intact lipophorin. Following induction by various elicitors, including sub-lethal doses of Bt-formulation, plasma from both strains displayed a dramatic increase in the level of an 85 kDa protein (Fig. 3).

3.3. Cloning of p85 coding DNA

Since the p85 binds to Cry1Ac on Western blots (G. Ma, unpubl. data) and under native conditions (see below), we identified the protein by micro-sequencing. In-gel protein digestion and separation of peptides on an HPLC column produced several peptides. The following peptide sequences were determined: Peak number 14 (elution time 34.2 min) was L/H A/T E/P E/H IEVPH, peak number 26 (elution time 44.0 min) was LTDEPEGFPVNRPL, and peak number 37 (elution time 53.5 min) was NIEHYXXVVAVTY.

Cloning of the corresponding coding DNA (see Material and Methods) revealed a deduced protein of 706 amino acids with conserved hemocyanin M and C domains (Fig. 4). The first 19 amino acids were identified as a signal peptide with a signal peptide cleavage site at position 18(S) and 19(D), which predicts an extracellular location with several N-glycosylation sites and one possible O-glycosylation site.

3.4. Hexamerin interacts with toxin under native conditions

Since hexamerin binds to GalNAc-specific lectins (Fig. 3) and Cry1Ac on Western blots (G. Ma, unpubl. results), we examined whether this binding is also observed with native proteins. Plasma from induced larvae was mixed with activated Cry1Ac and separated into precipitating aggregates and soluble supernatant. When pellets were suspended and treated in various loading buffers at different temperatures, no soluble protein was recovered (not shown). We therefore analysed the supernatant for the reduction of proteins that may have interacted with Cry1Ac to form an insoluble aggregate. When protein extracts of plasma supernatants were analysed on SDS-PAGE, a concentration and time-specific reduction of hexamerin was observed in Cry1Ac-treated plasma, while the other major proteins, including prophenoloxidase, apolipophorin II and arylphorin were unaffected (Fig. 5).

Since hexamerin binds to the hexameric GalNAc-specific lectin from *Helix pomatia* (HPL) (Fig. 3), we explored whether this and other lectins induce aggregate formation as well. When plasma from induced larvae was mixed with Cry1Ac and lectins, only GalNAc-specific lectins (tetrameric *Vicia vilosa* (VVL) and hexameric HPL) caused aggregate formation, whereas the Gal-specific peanut agglutinin (PNA) had no effect (Fig. 6). Moreover, the

Gal/GalNAc-specific winged bean lectin (WBL), which is probably monomeric under the pH-conditions used, also showed no aggregation (Fig. 6). In this experiment mature Cry1Ac reacted like an oligomeric GalNAc-specific lectin.

3.5. Hexamerin is found in the gut

To test whether hexamerin is found in the gut we inspected gut protein extracts on Western blots using antibodies against recombinant hexamerin. A protein band corresponding to hexamerin in size was stained with the antibodies in extracts from susceptible and resistant larvae (Fig. 7). While extracts from susceptible larvae showed a single band, those from resistant larvae showed multiple bands, which could be due to protein degradation or protein modification. Further experiments are required to determine the cause of protein heterogeneity in the extracts from resistant larvae.

3.6. Mature Bt-toxin forms tetrameric complexes *in vitro*

Since these experiments indicated that oligomeric but not monomeric lectins cause aggregation of plasma proteins, we examined whether Bt-toxin existed in an oligomeric form outside lipid membrane-bilayers. When Cry1Ac crystal was processed for different time periods in the presence of gut juice extracts, high molecular weight complexes were observed at intermediate times. When digestion continued for longer time periods, a 60 kDa protein appeared in addition to the putative mature 69 kDa protein (Fig. 8a). Under conditions where mixtures of the two proteins co-existed, the high molecular weight complex was separated into several narrow bands above 250 kDa (Fig. 8a, arrows). The relative distribution of these high molecular weight complexes correlated with the relative amounts of the 60 and 69 kDa proteins, which is consistent with the formation of hetero-tetramers of 60 and 69 kDa proteins. Both the complexes and the 60 and 69 kDa proteins stained with anti-toxin antibodies (Fig. 8b). The tetrameric complexes were stable in SDS at 65°C but reverted to low molecular weight monomers at 100°C (Fig. 8b). A similar complex was observed when the 130 kDa toxin precursor was incubated with trypsin in a lipid-free buffer (not shown).

This suggests that Cry1Ac-toxin exists as a tetrameric complex with GalNAc-specific lectin properties, which can interact with soluble glycoproteins to form detergent-insoluble aggregates.

3.7. Mode of inheritance of Bt-tolerance

To investigate a possible maternal effect, we performed two crosses, one where females from the resistant population were individually crossed with susceptible males (RxS), and a reciprocal cross, where susceptible females were crossed with resistant males (SxR). One possible mode of transmission could be the incorporation of maternal immune-components into the egg, acting as immune-elicitors during embryogenesis and priming the neonates to acquire an elevated immune-status. If this assumption is correct, genetic crosses between resistant and susceptible insects should be non-reciprocal; females from resistant populations mated with susceptible males will produce immune-induced offspring, whereas resistant males and susceptible females will produce susceptible offspring. In contrast, if resistance were based on an embryonic phenotype caused exclusively by zygotic gene expression, resistance in F_1 offspring would only be expected if the resistance gene locus were linked to a sex chromosome, or was semi-dominant (see Material and Methods).

Analysis by Cox's proportional hazard model showed that the hazard ratio was significantly lower for the RxS larvae compared to the SxR larvae (Table 1A), and correspondingly the contribution of the resistant female was greater than the resistant male (Table 1B). These results are consistent with a maternal effect resulting in the transmission of an elevated immune status from the female to her offspring. The fact that the risk ratio for RxR was lower than for RxS and that the risk ratio for SxS was higher than SxR indicates an early zygotic contribution to the phenotype in addition to a maternal effect.

4. Discussion

A number of observations suggest that the observed Bt-resistance in a *H. armigera* strain is based on an elevated immune status. First, both gut extracts and plasma from the resistant strain displayed a higher melanization rate compared to the susceptible strain. Second, an inducible immune protein

in the hemolymph plasma was identified as hexamerin, which acts as a storage protein and pro-coagulant (Scherfer et al., 2004). The protein is also found in the gut and may be modified in the resistant strain. The protein bound to Cry1Ac and GalNAc-specific lectins to form an insoluble aggregate. Third, reciprocal crosses of resistant and susceptible insects revealed the transmission of the tolerance from one generation to the next by a maternal effect. As discussed for *E. kuehniella*, the most straightforward explanation for the observations is the incorporation of an immune-elicitor into the oocyte by an immune-induced female (Rahman et al., 2004). The elicitor can interact with embryonic tissues to induce the immune system of the neonate. By the time the neonate starts feeding, the insect is already induced thus increasing the chances of surviving the toxin. In turn, older larvae that are able to survive the initial exposure to Bt long enough on little or no food for immune induction to occur may continue to feed on contaminated food. This can explain the puzzling observation that susceptibility to Bt-toxin decreases in later stages of larval development.

That resistance is acquired in the embryo by an immune induction is a strong indication that the mutation in the resistant strain is not directly involved in altering the receptor-coding region. Although we cannot rule out a semi-dominant effect of a receptor mutation, from the hazard ratio data (Table 1b) its contribution to the overall tolerance would be relatively small. This was corroborated by the inspection of several aminopeptidase N genes from the resistant strain, which did not reveal any mutational changes that could be responsible for the observed tolerance (C. Angelucci and R. Akhurst, unpubl. data). In addition, the observation that hexamerin was inducible in both susceptible and resistant strains suggests that gene expression is not affected in the resistant strain and that the induction of the hexamerin gene is necessary but not sufficient to confer resistance. It is therefore likely that immune-specific effects, such as post-translational modifications and/or tissue-specific protein translocations, are involved in resistance in this system. The binding of GalNAc-specific oligomeric lectins to induced hexamerin suggests that glycosylation may be a prerequisite for the observed aggregation reaction. Although there are other mechanisms (Banks et al., 2001), crystal toxins may use GalNAc to bind to aminopeptidase receptors at

the brush border membrane (Burton et al., 1999; Derbyshire et al., 2001). This opens up a number of scenarios for toxin interactions in the gut lumen. Hexamerin, like other immune proteins, may be secreted into the gut lumen in a GalNAc-glycosylated form that interacts with the mature toxin to form insoluble aggregates. In addition, since the mature toxin was shown to be oligomeric, a requirement for the formation of aggregates, this suggests that the active form of the toxin is oligomeric. Finally, if GalNAc-specific glycosylation is modified as a result of immune induction, the target proteins may not be restricted to pro-coagulants but include other proteins, such as aminopeptidases, which are know to have GalNAc-specific O-glycosylation (Burton et al., 1999).

Post-translational modifications of receptors and pro-coagulants may be compatible with previously reported observations (Akhurst et al., 2003; Liao et al., 2002), including changes in receptor-binding of the toxin in the absence of any overt mutations in candidate aminopeptidase N genes, and possible interactions with pro-coagulants reported in this paper. Such pleiotropic effects are consistent with a model of resistance or tolerance to the toxin based on inducible gene functions that perform post-translational modification of multiple proteins, rather than rare mutations in the receptor gene.

The nature of the elicitor function in the ovary that leads to maternal transmission of the immune status is not known. It may involve either an unknown ovarian molecule that is modified by the immune induction, or hemolymph proteins that are incorporated into the ooplasm as storage proteins. Being an egg storage protein (Terwilliger, 1999) that is transported from the hemolymph into the ooplasm during vitellogenesis (Burmester et al., 1998), a modified form of hexamerin is a potential candidate. However further experiments are needed to identify the elicitor and its role in the transmission of tolerance to the toxin.

Whatever the molecular basis, the similarities in the findings for *E. kuehniella* (Rahman et al., 2004) and *H. armigera* (this report), where Bt-tolerance is associated with an elevated immune response and can be transmitted to offspring by a maternal effect suggest that the capacity for species to develop Bt-tolerance through immune-related processes could be common. These observations may have significant implications in the field. Although the

observed levels of immune-related tolerance are low compared to those associated with receptor mutations, if low-level tolerance is due to favourable combinations of pre-existing alleles rather than rare mutations then it may play an important role as a first step to the development of high-level resistance. The transmission of immune status and tolerance to the next generation by a maternal effect may act to reduce the loss in genetic variation otherwise associated with selection, facilitating the emergence of more highly Bt-tolerant insects that in turn may survive in sufficient numbers under continued selection pressure to develop mutations that genetically fix the elevated immune status or alter receptor function (Morin et al., 2003).

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Tables:

Table 1. Hazard ratios from a 7-day Bt-bioassay, determined by Cox's regression using Efron's (1977) partial likelihood method, with the SxS cross treated as the baseline hazard (i.e. hazard ratio of 1). **A)** analysed for the crosses of resistant and susceptible *H. armigera* strains. **B)** analysed for male and female of the resistant *H. armigera* strain.

Table 1A

Cross (Female x Male)	Hazard Ratio	95% CI	P
RxR	0.041	0.026 - 0.067	<0.0001
RxS	0.183	0.134 - 0.25	<0.0001
SxR	0.554	0.442 - 0.694	<0.0001

Table 1B

Sex	Hazard Ratio	95% CI	P
Male	0.472	0.368 - 0.577	<0.0001
Female	0.131	0.101 - 0.170	<0.0001

Figure legends:

Fig. 1 Typical melanization reactions measured as the relative absorbance at 490 nm over 30 min. A) Cell-free hemolymph (plasma) from groups of five 3rd instar resistant and susceptible caterpillars diluted in PBS-solution. Note the slight reduction in relative absorbance observed in plasma from the resistant strain after 10 mins due to coagulation reactions. B) Gut protein extracts from resistant and susceptible caterpillars diluted in PBS-solution containing 100mM DOPA.

Fig. 2 Blackening of the peritrophic membrane and gut of resistant larvae. The grey appearance of the surface of epithelial cells in resistant larvae may be the result of melanization by hemolymph-derived phenoloxidase of components attached to the basement membrane. In addition black spots and dot-like structures were visible in the gut lumen and at the peritrophic membrane from resistant caterpillars only. Gut epithelial cells were not visibly different.

Fig. 3 SDS polyacrylamide gels of cell-free hemolymph (plasma) from induced (I) and non-induced (C) larvae. **A**) Gel stained with Coomassie blue. A protein band at 85 kDa is visible in plasma from induced larvae, in addition to a band at around 160-180 kDa that is not always visible and may constitute a dimer. **B**) Western blot incubated with peroxidase-conjugated GalNAc-specific HPL and anti-arylphorin antibodies visualised with peroxidase-conjugated secondary antibodies. The protein band labelled by anti-arylphorin antibodies is comprised of three proteins with similar sized apolipophorin II and prophenoloxidase proteins in addition to arylphorin.

Fig. 4 Hexamerin sequence and comparison. **A**) Domain structure of hexamerin from *H. armigera*. **B**) Sequence comparison of hemocyanin C domain of hexamerin. Similar results were obtained with hemocyanin M domain (not shown).

Fig. 5 Toxin forms insoluble aggregates with hemolymph protein. SDS polyacrylamide gel, stained with Coomassie blue, of cell-free hemolymph (plasma) from induced larvae incubated with proteolytically activated Cry 1Ac for various time periods. Since the toxin formed insoluble aggregates, the plasma supernatant was analysed for the absence of proteins. In this experiment 2µg of activated Cry1Ac was incubated with plasma for 10, 20 and 30 min at RT, then centrifuged for 1min at 12000rpm. PBS treated plasma is shown as a negative control. Note that the triple protein band at 75 kDa is unchanged, whereas the amount of p85 is reduced. There is also a slight reduction in the band at around 160-180 kDa, which could be a dimer of p85. The identity of the band(s) around 64 kDa is not known.

Fig. 6 Toxin and lectins interact with hemolymph hexaerin. SDS-PAGE (12% gel) stained with Coomassie blue of ca. 5µg aliquots of cell-free hemolymph (plasma) from induced larvae incubated with various lectins and with Cry1Ac, showing enhanced aggregation and precipitation of hemolymph components, including p85, after incubation of plasma with oligomeric GalNAc-specific lectins and Cry1Ac. VVL=*Vicia vilosa*, a tetrameric GalNAc-specific lectin; Cry1Ac (gut juice-activated Bt-toxin); PNA=peanut agglutinin, a tetrameric Gal/GalNAc-specific lectin; HPL=*H. pomatia*, a hexameric GalNAc-specific lectin; WBL=winged bean lectin *Psophocarpus tetragonolobus,* a monomeric GalNAc-specific lectin; C=PBS control. Addition of gut-juice extracts at a concentration equivalent to those used in toxin activation did not have an effect and proteins were similar to the PBS control (not shown). In this preparation little or no 160-180 kDa protein was detected.

Fig. 7 Hexamerin is found in the gut. Midgut tissue and content from resistant (ISOC4) and susceptible (ANGR) 5th instar larvae respectively were separated and Western blots analysed using antibodies against recombinant hexamerin domain C as a probe. .

Fig. 8 Mature toxin forms a stable tetramer *in vitro*. SDS-PAGE (7.5% gels) of activated Cry1Ac toxin following incubation of protoxin with gut juice extracts for different time periods and extraction at either 65 or 100°C. Protoxin was purified from *B. thuringiensis* subsp. *kurstaki* HD-73 and solubilized in a

solution containing 30 mM Na_2CO_3 and 1% mercaptoethanol at pH 9.6. Western blots were incubated with Cry1Ac-specific antibodies and then visualised with peroxidase-conjugated secondary antibodies. **A**) Protoxin and gut juice-extract from the lepidopteran species *Pieris rapae* were incubated for 30 min (1), 1h (2), 2h (3) and five hours (4) and extracted at 65°C in SDS-containing buffer. The mature toxin (69 kDa) is the predominant band initially (large arrow), but is replaced by a 60 kDa overdigested protein (large arrowhead). Both proteins appear to form hetero-oligomeric complexes, which form a cluster of narrow bands above the 250 kDa marker band (small arrows). The relative amounts and distribution of these narrow bands are correlated with the relative composition of the 60 and 69 kDa bands. **B**) Gut juice-activated toxin after incubation of one hour (1) and five hours (2), marker (M), gut juice activated toxin (as in 1) extracted at 100°C. Note extraction at 100°C in SDS-containing buffers eliminated the bands above 250 kDa. Cry1Ac-specific antibodies were visualised with peroxidase-conjugated secondary antibodies.

Fig. 1A

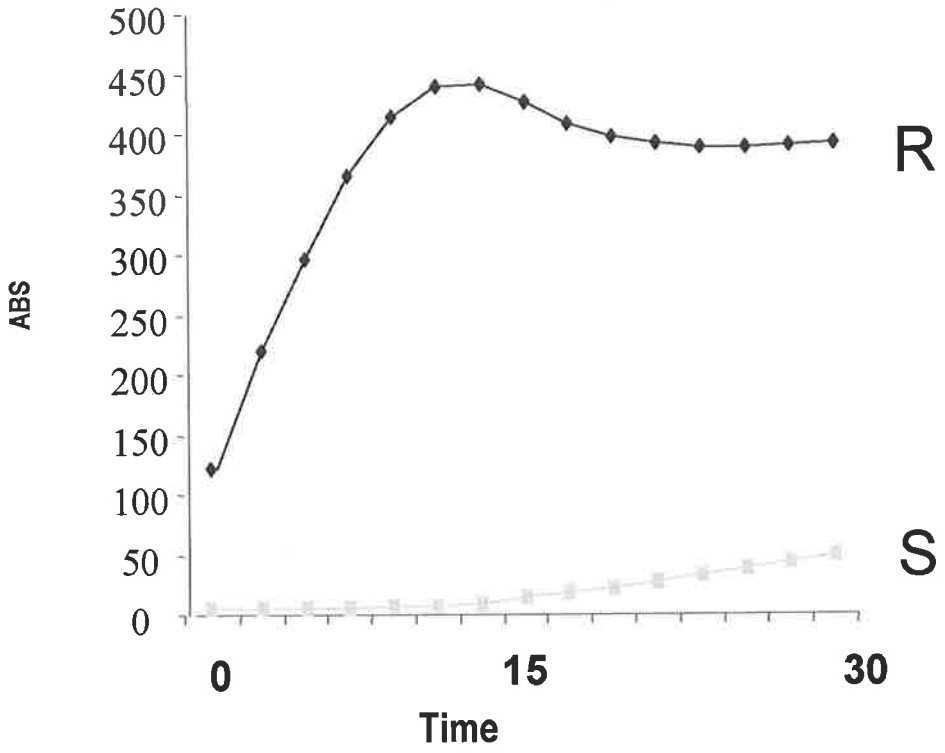
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Fig. 1B

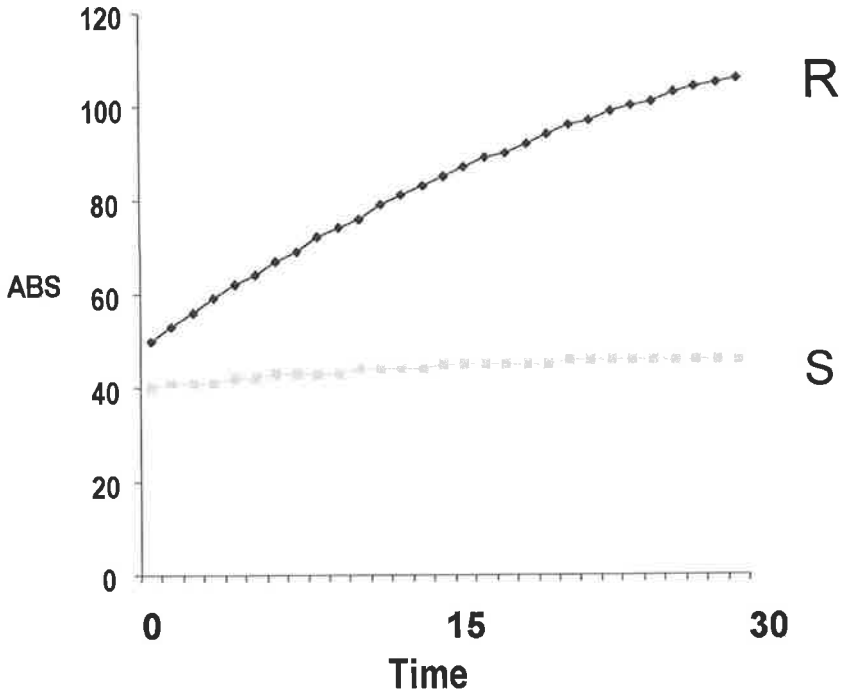
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Fig. 2

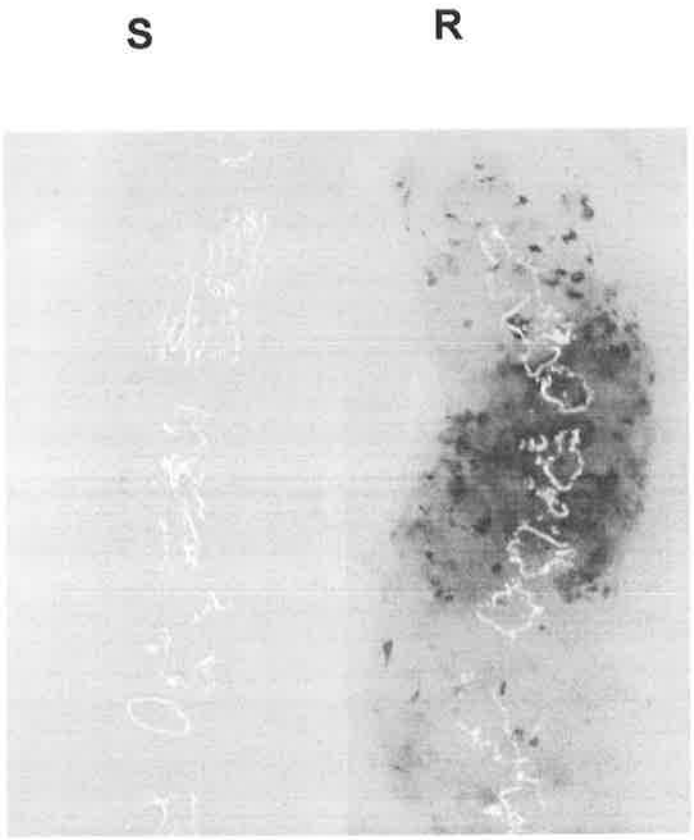
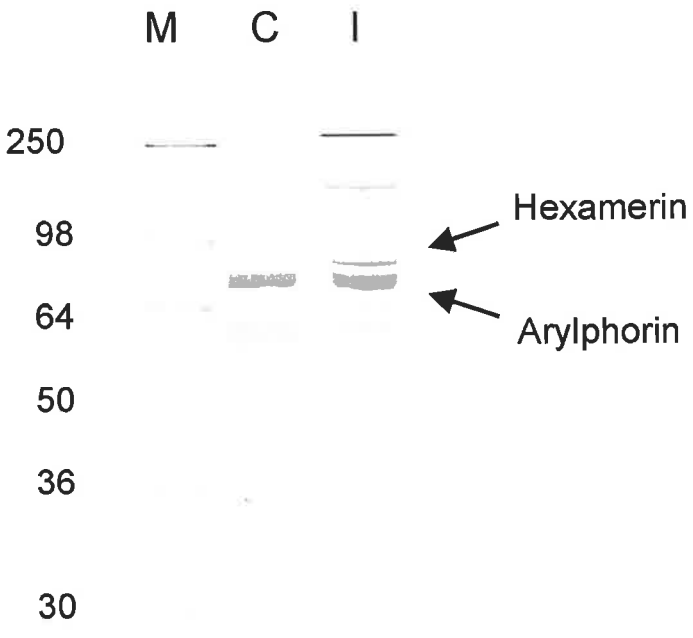


Fig. 3A



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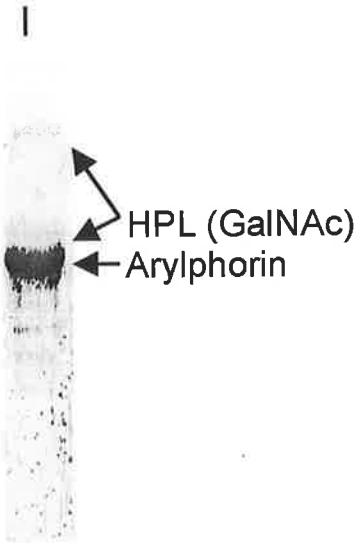
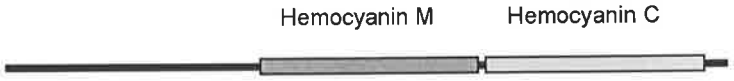


Fig. 4A



Hemocyanin M (copper-containing domain)

Fig. 4B

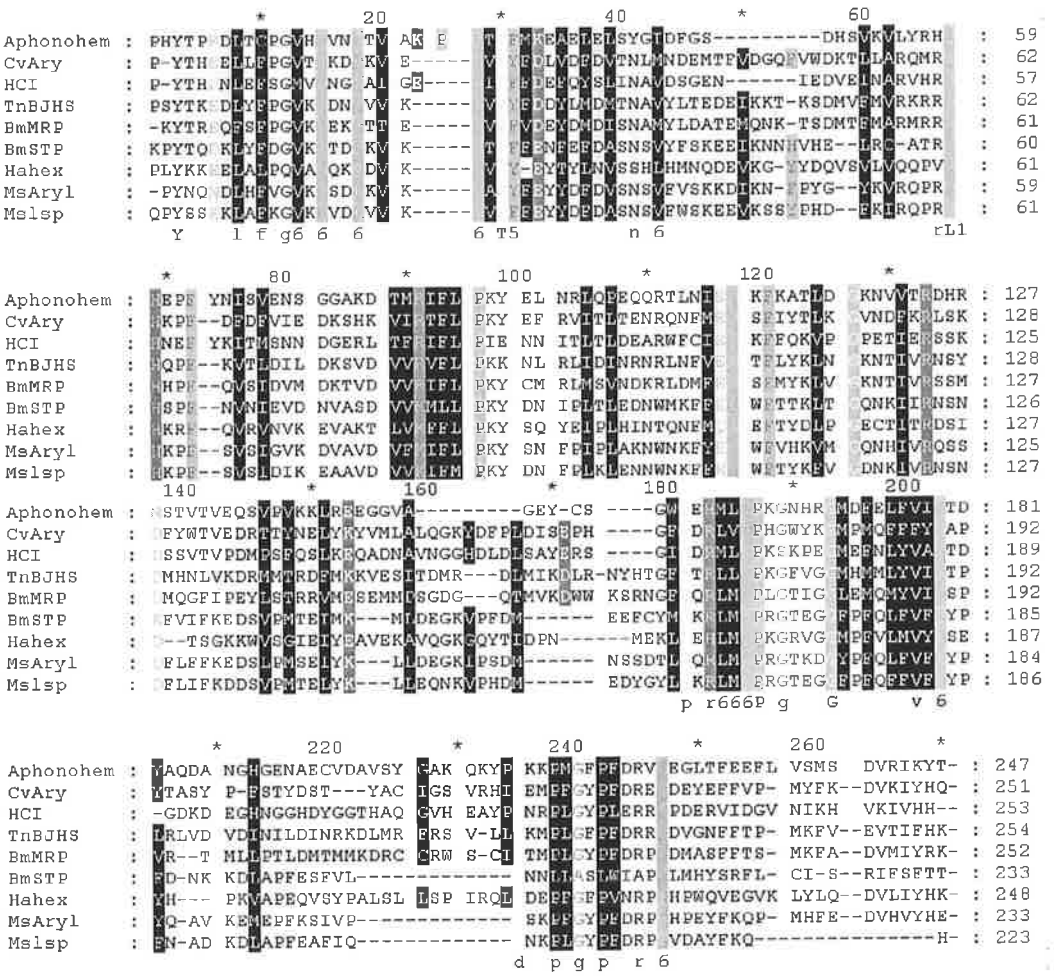
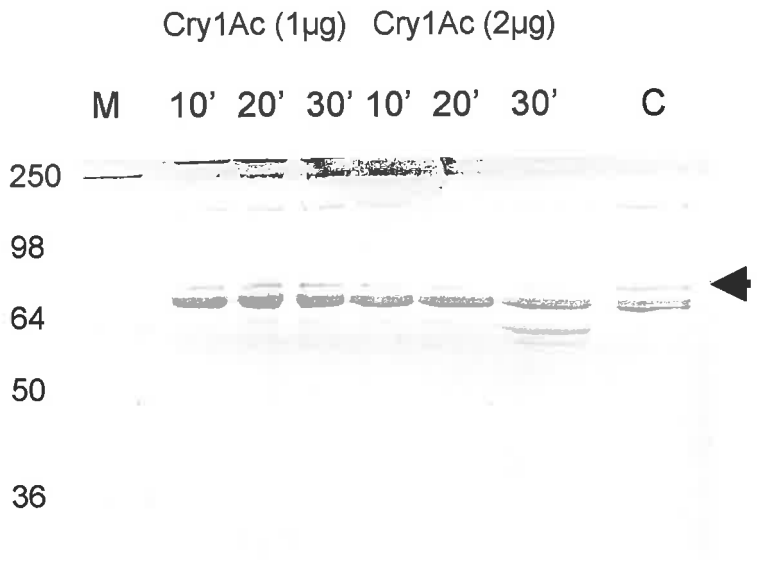


Fig. 5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Cry1Ac (2µg)
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Fig. 6

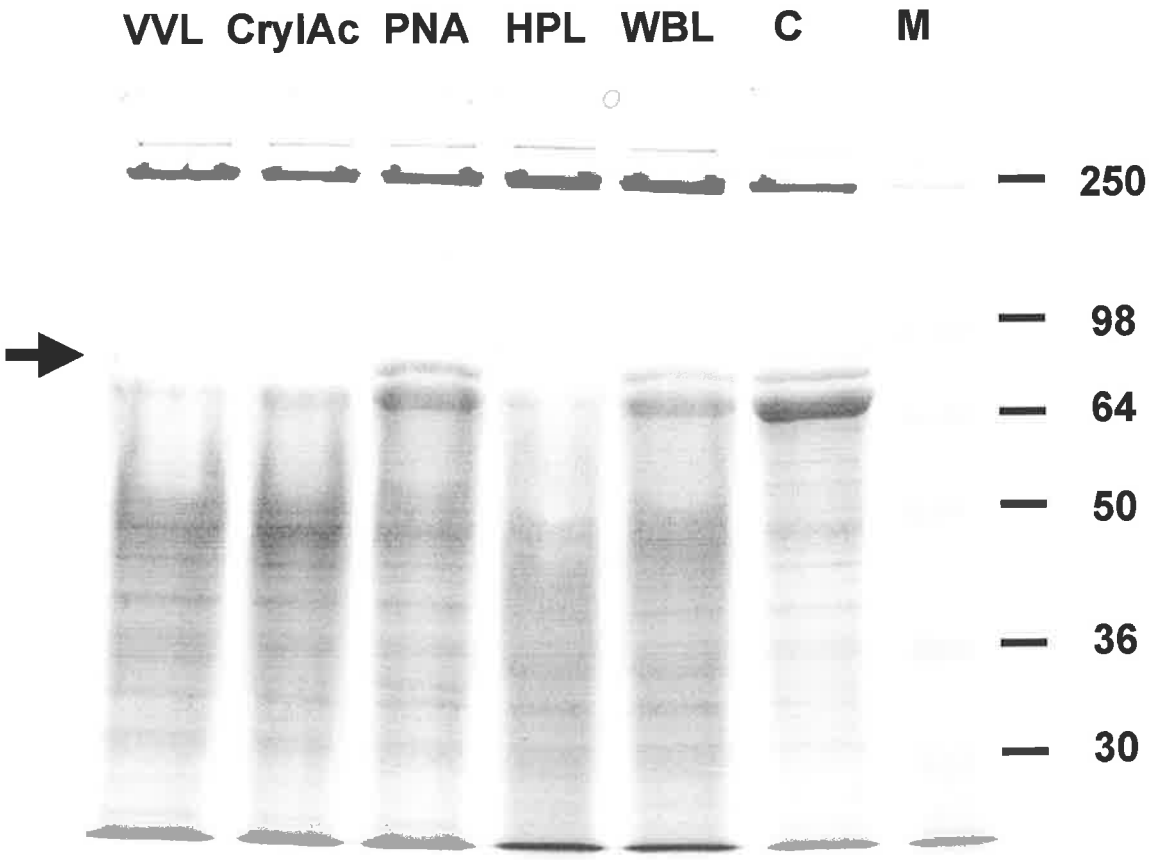
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Fig. 7

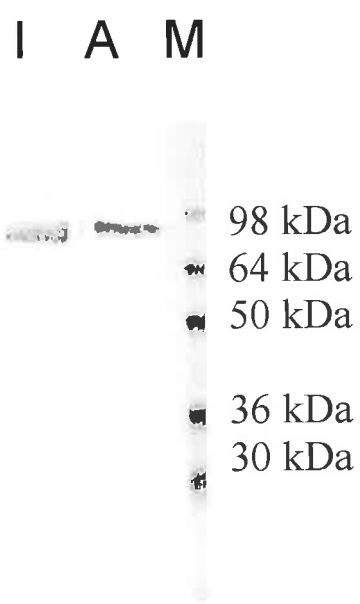


Fig. 8

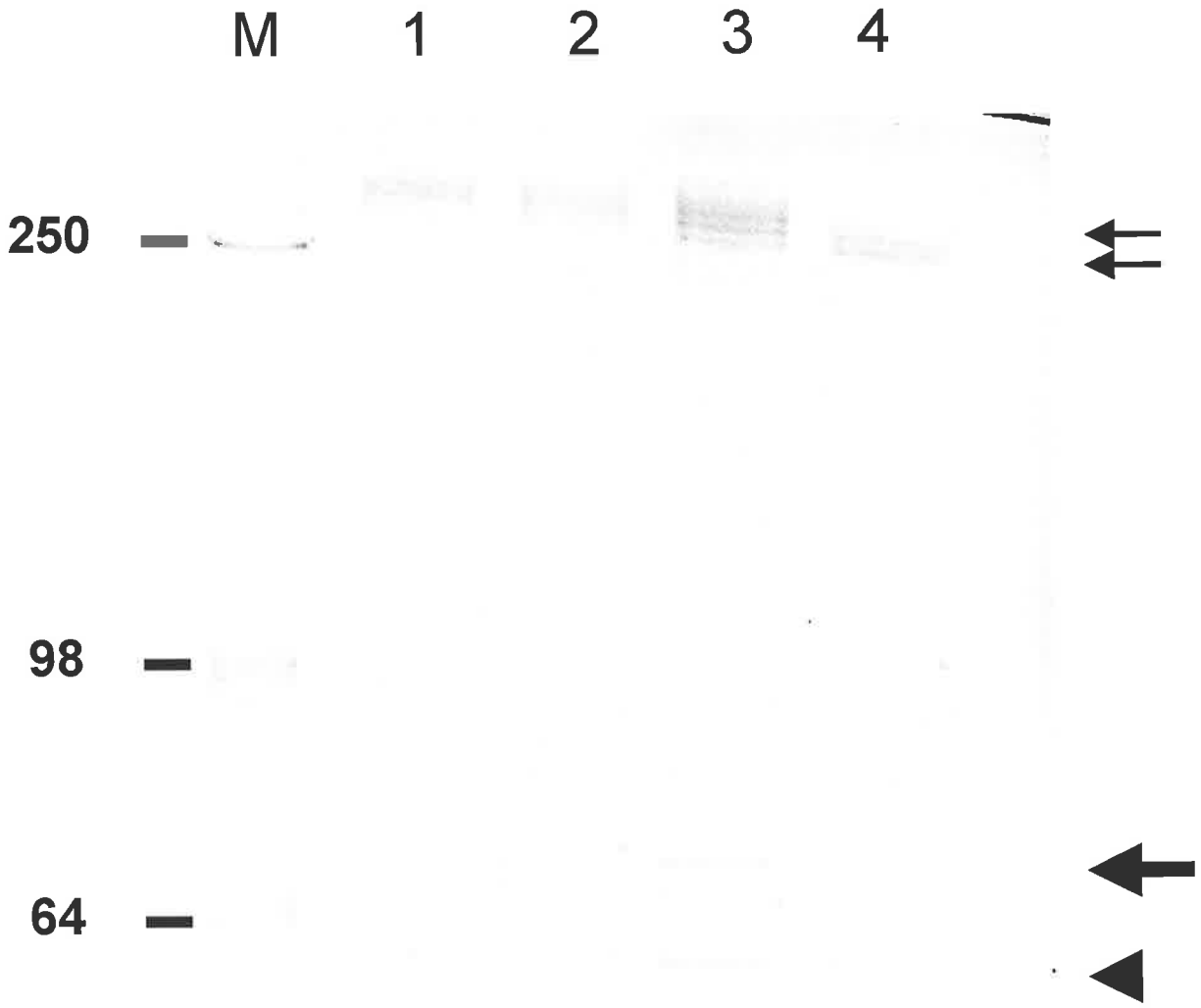
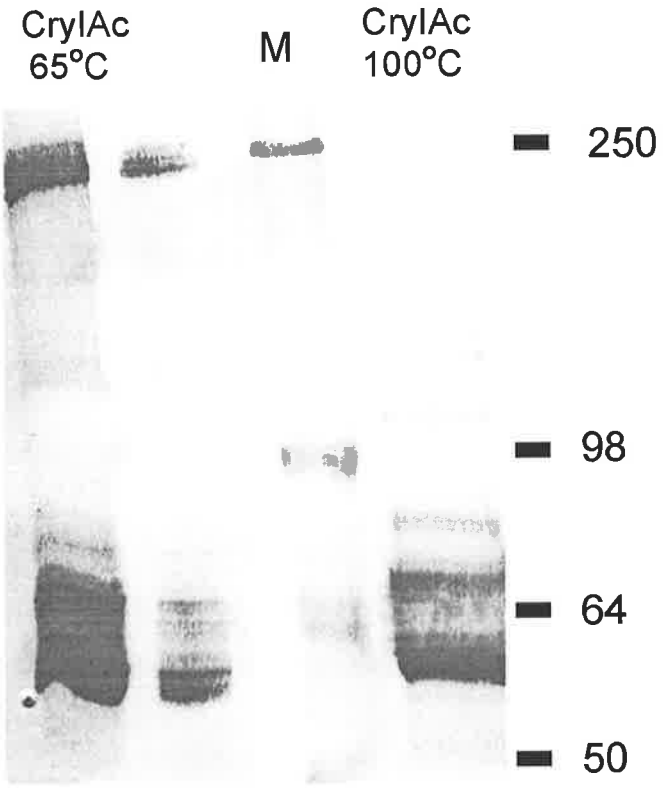


Fig. 7B



Appendix 1E.

Lectin-induced hemocyte inactivation in insects

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Lectin-induced haemocyte inactivation in insects

Richard Glatz[a], Harry L.S. Roberts[a], Dongmei Li[a], Muhammad Sarjan[b], Ulrich H. Theopold[c], Sassan Asgari[d], Otto Schmidt[a,*]

[a]*Insect Molecular Biology, School of Agriculture, University of Adelaide, Glen Osmond, SA 5064, Australia*
[b]*Faculty of Agriculture, University of Mataram, Lombok, Indonesia*
[c]*Department of Molecular Biology and Functional Genomics, Stockholm University, S-10691 Stockholm, Sweden*
[d]*Department of Zoology and Entomology, School of Life Sciences, University of Queensland, St Lucia QLD 4072, Australia*

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Abstract

Most multimeric lectins are adhesion molecules, promoting attachment and spreading on surface glycodeterminants. In addition, some lectins have counter-adhesion properties, detaching already spread cells which then acquire round or spindle-formed cell shapes. Since lectin-mediated adhesion and detachment is observed in haemocyte-like *Drosophila* cells, which have haemomucin as the major lectin-binding glycoprotein, the two opposite cell behaviours may be the result of lectin-mediated receptor rearrangements on the cell surface. To investigate oligomeric lectins as a possible extracellular driving force affecting cell shape changes, we examined lectin-mediated reactions in lepidopteran haemocytes after cytochalasin D-treatment and observed that while cell-spreading was dependent on F-actin, lectin-uptake was less dependent on F-actin. We propose a model of cell shape changes involving a dynamic balance between adhesion and uptake reactions.

Keywords: Lectin; Adhesion; Counter-adhesion; Endocytosis; Actin-cytoskeleton; Cytochalasin D; Glycodeterminants

1. Introduction

Soluble counter-adhesion molecules, such as thrombospondins (Chen et al., 2000), SPARC, (Yan and Sage, 1999) and tenascin (Midwood and Schwarzbauer, 2002), destabilize cell–matrix contacts by inhibiting focal contact formation and assembly and prevent cell adhesion to glass or fibronectin substrates. Conversely, immobilised counter-adhesion molecules promote adhesion and spreading (Bornstein, 2001) in ways that are different from focal contacts (Adams, 1995). Although the mode of action of vertebrate counter-adhesion molecules and similar molecules in insects (Adams et al., 2003) is not known, the assumption is that immobilized and soluble counter-adhesion molecules interact with different receptors invoking distinct signalling pathways (Chandrasekaran et al., 2000), depending on the exposure of cells to the immobilized or soluble form (Adams, 1995; Goicoechea et al., 2002).

We and others have noticed that some oligomeric lectins act as adhesion molecules by promoting spreading on an artificial surface, but on different substrate conditions act as counter-adhesion molecules by detaching already spread cells. For example, the pioneering work of the Rizki's demonstrated that lectins cause spreading of *Drosophila* cells on a glass surface and cause cell fusion of neighbouring cells (Rizki et al., 1975). Similarly *Drosophila* haemocyte-like cells (and other primary cells as well) will spread more extensively when plated on immobilised lectins (Rogers et al., 2003). However, under some conditions spread cells that are exposed to soluble lectins detach and round up. Again this was first detected in fat body cells, where detachment from tissue-contacts and associated

*Corresponding author. Tel.: +618-8303-7252; fax: +618-8303-7109.
E-mail address: otto.schmidt@adelaide.edu.au (O. Schmidt).

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rearrangements of actin-cytoskeleton was observed after lectin applications (Rizki and Rizki, 1983). Similar to oligomeric lectins, the hexameric apolipophorin III shows increased phagocytosis when immobilised onto particles and detachment of spread hemocytes when applied in soluble form (Whitten et al., 2004).

Recently, we observed another lectin-mediated response in *Drosophila* haemocyte-like cells, with *mbn-2* cells (displaying GalNAc- and Gal-containing haemomucins (Theopold et al., 1996, 2001) responding to the presence of two soluble oligomeric lectins, the GalNAc-specific *Helix pomatia* lectin (HPL) and the Gal-specific lectin from *Arachis hypogaea* (peanut agglutinin, PNA), by undergoing lectin-induced uptake reactions (U. Theopold, unpublished results). These reactions are different from actin-dependent constitutive macropinocytosis (Johannes and Lamaze, 2002). While constitutive macropinocytosis is dependent on actin-mediated membrane ruffling (Anton et al., 2003), creating receptor-depleted vesicles (Swanson and Watts, 1995), lectin-mediated uptake reactions appear to be actin-independent and occur after receptor-clustering, resulting in vesicles that are receptor-rich. On the assumption that haemomucin is the only glycoprotein receptor on the surface of *Drosophila* cells (Theopold et al., 2001), receptor–lectin interactions leading to adhesion must be different from those leading to lectin uptake.

A similar conundrum exists in polarised cells, where impairment of actin-containing microfilaments by cytochalasin D, a fungal actin-capping protein (Cooper, 1987), selectively inhibits the capacity of cells to endocytose membrane-bound and fluid-phase markers applied to the apical surface, without affecting endocytosis from the basolateral surface (Gottlieb et al., 1993). The authors concluded that 'the ankyrin-mediated linkage of some basolateral membrane proteins to the underlying cytoskeleton, which is triggered by the establishment of cell–cell contacts, appears to prevent the endocytosis of those proteins and thus contribute to their metabolic stabilisation'. Since then numerous examples have been found in polarised epithelial cells, in motile cells and during cell-spreading, where the role of actin-cytoskeleton appears to be counter-intuitive (Woodring et al., 2003). For example, c-Abl seems to have a negative role in cell migration but positively contributes to filopodia formation, membrane ruffling and neurite extension (Woodring et al., 2003). Likewise, the Ena/VASP proteins decrease cell motility yet positively regulate actin polymerisation (Krause et al., 2002). This suggests that actin may have functional roles in cell shape changes other than acting as a motor protein.

The observation that lectins mediate cellular uptake reactions and cause adhesion by interacting with glycoprotein receptors implies that lectins act like counter-adhesion molecules. To examine whether these seemingly opposite reactions are the result of a dynamic balance of interactions, involving lectin-mediated receptor-internalisation and actin-mediated receptor-stabilisation on the cell surface, we analysed the effect of lectins on actin-cytoskeleton in live haemocytes.

2. Material and methods

2.1. Insects

We used lepidopteran haemocytes, which readily spread on glass surfaces and remain viable in insect ringer for up to two days without visible damage. They contain two major cell types: plasmatocytes, which spread quickly on artificial surfaces acquiring a fried egg shape, and granulocytes, which remain round-shaped but release filopodia (see Fig. 1). Most experiments were

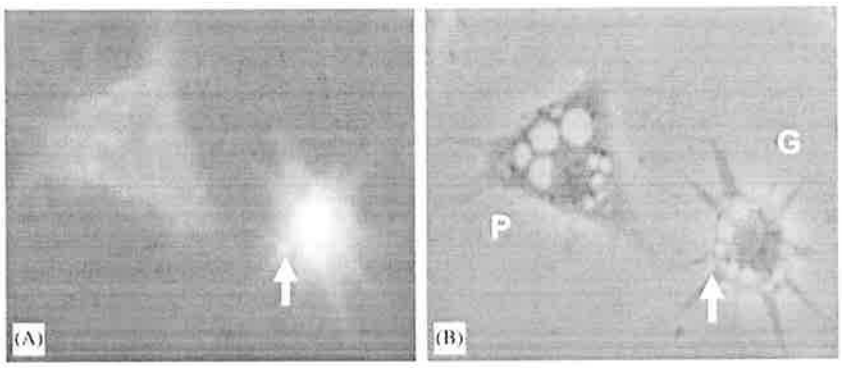

Fig. 1. Lectin-mediated clustering in insect haemocytes from *H. armigera*. (A) Live haemocytes from *H. armigera* larvae were spread on a glass surface and incubated with a buffer solution containing FITC-conjugated GalNAc-specific *H. pomatia* lectin (HPL) and inspected under indirect FITC-eliciting UV-light. Clustering of fluorescence stain was visible in plasmatocytes (P) and granulocytes (G) after a few minutes (arrows), long before detachment is observed (several hours). After a few hours the fluorescence was visible inside large endosomal vesicles (see below) next to non-stained macropinocytosis vesicles (arrowheads), which formed before spreading, possibly by membrane 'ruffling' as part of a constitutive macropinocytosis (Johannes and Lamaze, 2002). (B) Corresponding phase contrast picture.

repeated in more than one lepidopteran species to indicate that the outcomes are not species-specific. Here we show the results from three species, *Helicoverpa armigera*, *Pieris rapae* and *Galleria mellonella*.

H. armigera larvae were reared on artificial diet (Akhurst et al., 2003) under an L14:D10 light-dark regime. *P. rapae* larvae were kept on fresh cabbage leafs on a daylight/night cycle. *G. mellonella* larvae were kept on artificial diet in the dark.

2.2. Haemocyte preparations

Fourth instar larvae were bled into non-supplemented Grace's insect medium (GIM) saturated with phenylthiourea (PTU), via removal of a proleg, and the haemolymph centrifuged at 2300 × *g* for 5 min at RT. Supernatant (cell-free haemolymph) was removed and the cellular pellet resuspended in GIM. 40 µl of haemocyte resuspension was added to slide wells and the cells allowed to spread for 15 or 30 min.

For lectin-treatment in suspension, haemocytes were diluted in buffer with and without lectin in Eppendorf tubes completely filled with buffer in the absence of air bubbles and kept in suspension with light rotations. Buffer solutions included GIM or phosphate-buffered saline (PBS) solutions (138 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 7.3 mM Na_2HPO_4, pH 7.6), which gave similar results for treatments of up to 12 h duration at room temperature.

2.3. Haemocyte spreading

Haemocytes spread on artificial surfaces by attaching to extracellular-matrix-like protein secretions (Fogerty et al., 1994; Gullberg et al., 1994), which are stored in vesicles and discharged in the course of haemocyte preparations or during the process of settling on the surface. Haemocytes attached more readily to artificial surfaces that were pre-treated with cell-free haemolymph (plasma). When plasma-treated surfaces were incubated with lectin and washed repeatedly, haemocytes formed large extensions, similar to *Drosophila* haemocyte-like cells spreading on immobilised lectin (Rogers et al., 2003). Preliminary experiments suggested to us that immobilised lectins cause spreading, whereas soluble lectins cause detachment. Given that haemocytes release lectin-binding substrates, lectin-treatment of spread haemocytes constitutes a mixture of the two opposite reactions. To observe detachment of spread haemocytes in the presence of soluble lectin, haemocytes were carefully washed to remove plasma without causing haemocyte aggregation. Attachment and spreading was performed in multitest glass slides (8 wells, ICN Biomediacals) and monitored under an inverted microscope. Since the time required for attachment and spreading of haemocytes varied between individual larvae from 1 to 5 h, haemocytes from three to five 4th instar larvae were isolated in a single batch and all treatments (lectin, cytD, lectin + cytD, and control) were performed with aliquots from the same batch in at least five independent experiments. Because granulocytes show spreading by extending filopodia (Fig. 1), which are sometimes difficult to detect in live cells, only plasmatocytes were monitored for spreading and detachment.

2.4. Haemocyte staining

Spread haemocytes were treated with FITC- or TRITC-conjugated lectins (GalNAc-specific hexameric *Helix pomatia* lectin (HPL, Sigma) at a concentration of 3 ng/µl at room temperature in darkness. After application of the lectin to live haemocytes, the lectin buffer was replaced with PBS containing 4% paraformaldehyde and after 15 min washed three times in PBS. For inspection of live haemocytes and internalisation of fluorescent dye, haemocytes were incubated in trypan blue (2 mg/ml GIM and filtered) to quench any surface fluorescence staining before inspection under indirect UV-light on an inverted or confocal microscope.

For inspection of actin-cytoskeleton, haemocytes were incubated in 4% paraformaldehyde and 0.5% Tween 20 for 15 min and washed three times before adding FITC-conjugated phalloidin (Sigma) for 30 min in darkness. After washing haemocytes three times in PBS, haemocytes were inspected under indirect UV-light.

2.5. Cytochalasin D treatment of spread cells.

Cytochalasin D (Sigma) was dissolved in 100% ethanol and diluted in GIM to a final concentration of 50 µg/ml, a concentration that kills less than 10% of *G. mellonella* plasmatocytes whilst completely inhibiting phagocytosis in treated cells (Vilcinskas et al., 1997b). Ethanol concentration (in GIM) was kept below 0.5% (v/v) a concentration that does not inhibit plasmatocyte-mediated phagocytosis (Vilcinskas et al., 1997a). Cell responses were dependent on cytochalasin D concentrations and the duration of treatments. For example, pre-treated haemocytes were precluded from attachment and spreading while already spread cells required treatment for up to an hour to detach (Vilcinskas et al., 1997a; our own obs). Likewise, phagocytosis is inhibited on spread cells at lower cytochalasin D concentration and before any signs of detachment from the glass surface can be detected (Asgari et al., 1997). Cytochalasin D concentrations were titrated to yield detachment of more than 90% of spread haemocytes from the glass surface within an hour.

Spread haemocytes were treated with 40 µl cytochalasin D in GIM, or GIM only, for up to 2 h at RT in

darkness. Cells were then washed three times exchanging GIM. FITC-labelled lectin (2 ng/μl) was then added to cells for 30 min in darkness and washed three times by exchanging GIM. Cells were then fixed for 20 min in 4% paraformaldehyde (in GIM) before exchanging medium three times. The same amount of trypan blue (0.2 mg/ml in GIM and filtered) was added to each well for 20 min at RT before exchanging GIM three times. Ten percent glycerol was applied to the slide and a cover slip placed over the wells. Cells could then be visualised under UV light.

3. Results

3.1. HPL can mediate clustering and uptake reactions

To exclude species-specific and cell-type-specific lectin-effects, such as those observed with plasmatocyte-specific spreading factors (Strand et al., 2000), we studied plasmatocytes and granulocytes from different lepidopteran species, and confirmed that lectin-effects were detected in all cell-types, including granulocytes and plasmatocytes. For example, when spread haemocytes and haemocyte-like cells were treated with fluorescence-labelled HPL, the first visible changes on the cell surface were small patches of labelling indicating clustering of receptors. These clusters were observed adjacent to large translucent vesicles (Fig. 1), which were already present at the time of spreading, created presumably by constitutive macropinocytosis (Johannes and Lamaze, 2002). After prolonged incubation with soluble HPL, lectins were internalised in vesicles at the sites of lectin clustering. After 4–6 h lectin-treated haemocytes from *G. mellonella* (Fig. 2) and *P. rapae* (not shown) showed a marked increase in the number of translucent vesicles compared to non-treated haemocytes. This indicates that lectin-mediated uptake reactions probably represent 'induced' macropinocytosis (Johannes and Lamaze, 2002). Since constitutive macropinocytosis reactions are dependent on actin-mediated membrane ruffling, this raises the question of whether lectin-induced uptake reactions are also dependent of actin.

3.2. Uptake of HPL can occur in the presence of cytochalasin D

It is well known that cell adhesion and spreading requires an intact actin-cytoskeleton (Rogers et al., 2003), with inactivation of F-actin causing detachment of cells or inactivation of haemocytes (Asgari et al., 1997). It also has been recognised for some time that actin-cytoskeleton plays a role in phagocytosis (Helantjaris et al., 1976), but the exact nature of that role is not clear. When spread haemocytes were treated with cytochalasin D, cells detached from the glass surface and acquired spindle-shapes (Fig. 3, cytD). At the concentration used in this experiment, cytochalasin D treatment caused more than 80% of plasmatocytes to detach within 30–60 min.

Similar to haemocyte-like cells, lepidopteran haemocytes that were treated with the oligomeric lectin HPL also detached and rounded-up, although less quickly and with fewer haemocytes acquiring spindle-form cell shapes compared to the cytochalasin D-treated cells (Fig. 3, HPL). The first signs of detachment after lectin-treatment were detected between 4 and 6 h (Fig. 2) and more than 80% of plasmatocytes were detached after 12 h, at which time less than 10% of non-treated cells had detached. This suggested to us that HPL interferes with cellular attachments to external binding sites resulting in the detachment of haemocytes. Given that HPL can cluster receptors and mediate uptake reactions, a possible explanation is that receptors at focal adhesion points are internalised by HPL despite cytoplasmic stabilisation through attachments to actin cables that form stress fibres at focal adhesion points. This implies that oligomeric lectin can potentially rearrange recep-

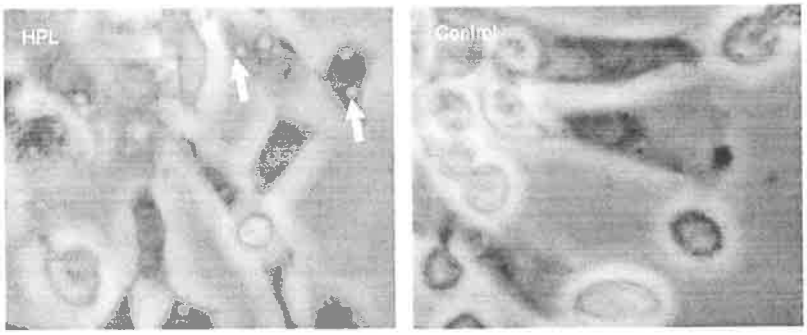

Fig. 2. HPL-induced uptake reactions in *G. mellonella* haemocytes. Lectin-treated haemocytes (HPL) showed more translucent vesicles (arrows) 4–6 h after the start of the treatment than non-treated haemocytes (Control). This coincided with the first signs of detachment (see Fig. 3). Note that *G. mellonella* haemocytes showed fewer vesicles at the start of treatment than *H. armigera* haemocytes (Fig. 1).

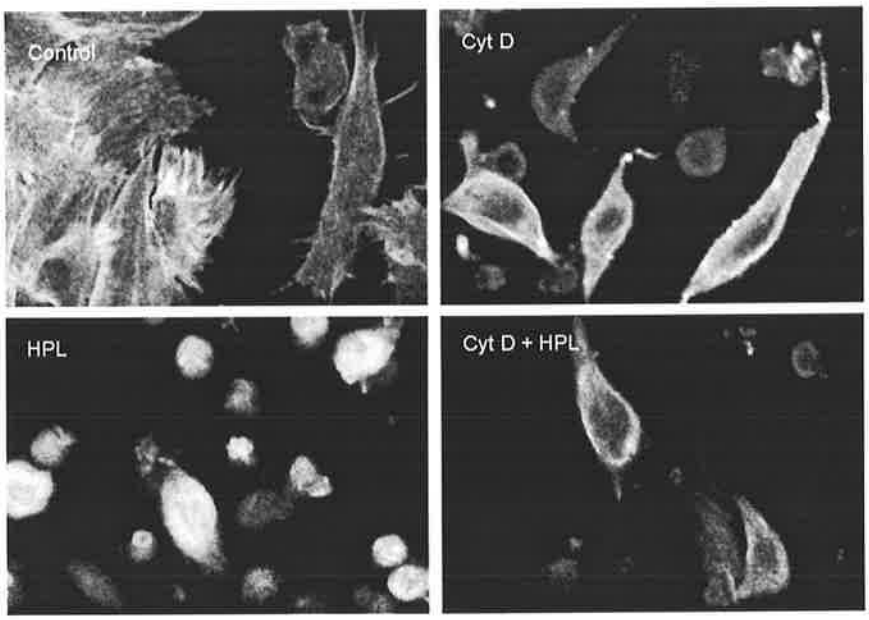

Fig. 3. Morphology and cytoskeleton changes of haemocytes from *P. rapae*. Haemocytes were allowed to spread on a glass surface and subsequently treated with Buffer only (Control), cytochalasin D (cyt D), *H. pomatia* lectin (TRITC-conjugated HPL, see Fig. 4) and cyt D and HPL combined (cytD + HPL). After treatment cells were quenched and fixed in the presence of non-ionic detergent and stained with FITC-conjugated phalloidin and inspected under indirect FITC-eliciting UV-light using confocal microscopy. Note the formation of stress fibres in spread cells, which were absent in treated cells. Both cytochalasin D and HPL-treated cells retreated from their attachment sites and formed round or spindle-formed shapes. Whereas cytochalasin D-treated cells accumulated actin at the periphery, HPL-treated cells showed irregular staining which was absent from cell extensions. Note that the combined treatment resembled cytochalasin D-treatment in cell morphology, which is in agreement with observations from separate treatments, where cytochalsin D-treatment revealed detachment effects after 30 60 min long before HPL-effects were first visible after 4 6 h (see Fig. 2).

tors on the cell surface and in the process destabilise their attachments to the actin-cytoskeleton.

To examine whether lectin-uptake can occur independently of F-actin, cytochalasin D-treated haemocytes were incubated with TRITC-conjugated HPL and inspected under a confocal microscope after quenching of the extracellular fluorescent dye by the addition of trypan blue. Inspection of median optical cellular sections showed irregular shaped endosomal vesicles mostly in the cortical region of the cell (Fig. 3, cyt+HPL; Fig. 4, arrows). Although the emerging endosomal vesicles remained at the cell periphery and were not transported to the cell interior, a process that may depend on actin-cables (Shupliakov et al., 2002), the actual formation of lectin-induced vesicles appears to be independent of F-actin.

Consistent with the suggestion that receptors can be internalised by lectins in the absence of intact cytoskeleton, when haemocytes that had been surface stained with HPL were treated with cytochalasin D the surface staining disappeared (see below). This raises the question of whether receptors on the cell surface require cytoplasmic stabilisation to prevent internalisation. In this context molecules acting like counter-adhesion

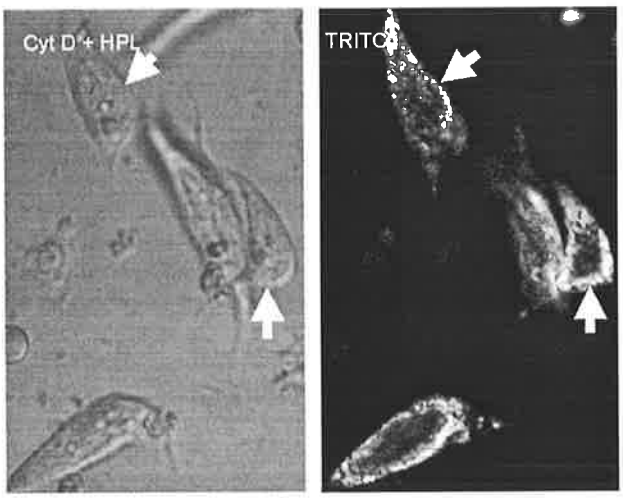

Fig. 4. HPL-internalisation in the presence of cytochalasin D. Haemocytes from *P. rapae* shown in Fig. 5 were examined in TRITC-eliciting indirect UV-light with TRITC-labelled HPL-uptake in the presence of cytochalasin D. Left is the phase contrast. The right picture shows haemocytes, where the optical section was adjusted through the centre of the cells, which showed small stained vesicles predominantly at the cortex, suggesting that HPL-mediated vesicle formation occurs in the presence of cytochalasin D, but that vesicles are not transported away from the cell cortex.

molecules, such as HPL, may force receptor-clustering and uptake reactions by overcoming actin-anchorage of surface receptors. Thus, excess HPL may destabilise the actin-cytoskeleton by overcoming receptor-stabilisation.

3.3. HPL can overcome F-actin anchorage of receptors

To examine the presence of cell surface receptors during lectin-mediated uptake and detachment, we treated live haemocytes with TRITC-conjugated HPL to monitor the uptake and detachment reactions. After haemocytes had detached, cells were fixed with paraformaldehyde and surface-stained with FITC-conjugated HPL. In these cells, there was a marked reduction in FITC-staining compared to non-treated spread cells. Only haemocytes that were surrounded by other haemocytes inside cellular aggregates were surface-stained (Fig. 5, FITC), with some of the FITC co-localising with TRITC-staining (Fig. 5, TRITC). This is consistent with the idea that HPL is able to induce uptake reactions by clustering glycoprotein receptors on the cell surface, and subsequently remove receptors from the cell surface by receptor internalisation. Since haemocytes inside cellular aggregates adhere to each other and retain receptors on the cell surface, this process appears to be delayed in those haemocytes that were surrounded by other haemocytes. The observations that internalised receptors were not recycled to the cell surface, or replenished by newly synthesised membrane vesicles, suggest that membrane transport within HPL-treated cells is impaired. Although HPL-treated cells showed a small amount of vesicle movement to the cell interior (Fig. 5), it was apparent that TRITC-stained vesicles remained mostly at the cell periphery and that lectin-binding receptors were eventually removed from the cell surface.

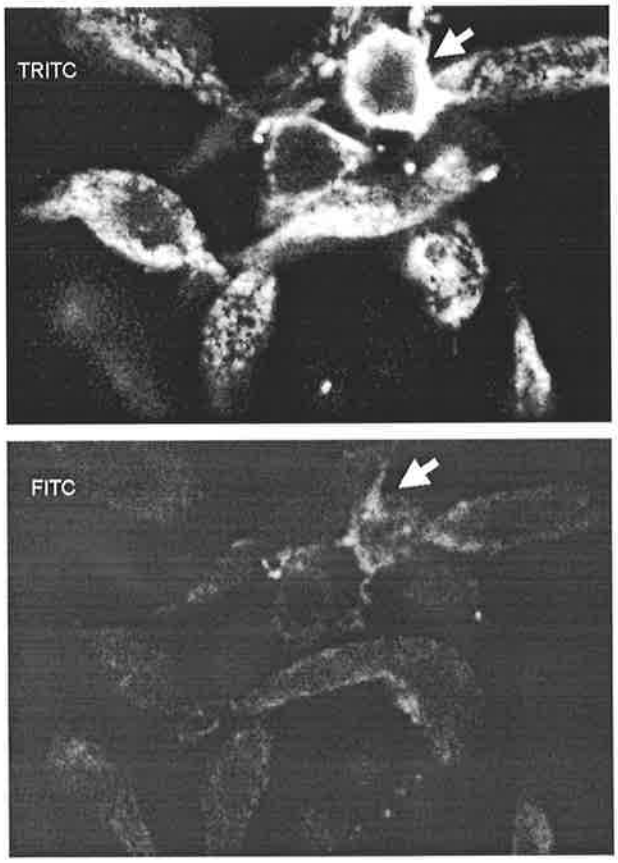

Fig. 5. Lectin-staining on the haemocyte surface of HPL-treated cells. TRITC-conjugated HPL was applied to spread *P. rapae* haemocytes. After detachment, which occurred 12 h after lectin-treatment, haemocytes were incubated with paraformaldehyde and stained with FITC-conjugated HPL to visualise residual lectin-binding to receptors on the cell surface. The picture shows a small haemocyte aggregate at the time of HPL-application, where one or two cells were surrounded by other haemocytes. HPL-uptake (TRITC) was visible in the surrounding haemocytes, which had spindle-formed cell-shapes, whereas haemocytes inside the aggregate were labelled on the surface (arrow) and showed attachment to underlying glass surface and spreading. HPL-surface staining (FITC) was reduced in cells at the periphery due to receptor-internalisation, but was somewhat higher inside the aggregate in cells that were surrounded by other cells at the time of treatment.

3.4. HPL can induce actin-depolymerisation

Since vesicle movement is impaired in HPL-treated cells (Fig. 5), we asked whether HPL-treatment can disrupt receptor-anchorage during uptake reactions and in the process destabilise the actin-cytoskeleton. We therefore exposed suspended *G. mellonella* haemocytes to HPL and compared attachment and spreading to suspended haemocytes in the absence of lectin. To minimise haemocyte aggregation, haemocytes were diluted into large volumes of buffer and kept in suspension by gentle rotation. Nonetheless, most cells formed aggregates in the presence of lectin and cells surrounded by other cells were able to retain receptors on the cell surface (see also Fig. 5). Only a small fraction of individual haemocytes were found in each preparation, which remained round with little contact to the surface. These cells showed partial actin-cytoskeleton breakdown with characteristic actin-aggregations around the nucleus (Fig. 6), which were not visible in the absence of lectin. This suggests that lectin-mediated uptake reactions in suspended haemocytes removed lectin-binding receptors from the surface and caused partial actin-depolymerisation. Similar to small cell aggregates found in spread cells (Fig. 5), aggregates formed during lectin incubation retained lectin-binding receptors on cells that were surrounded by other cells and were able to spread after making contact with the glass surface. In contrast, those cells on the periphery of aggregates appeared to have less cell-surface receptors and as a consequence were precluded from spreading.

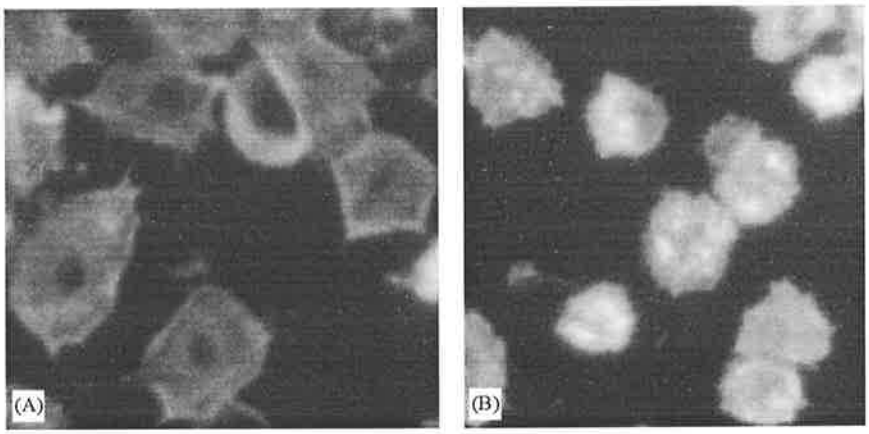

Fig. 6. F-actin and spreading after lectin treatment of haemocytes in suspension. *G. mellonella* haemocytes were suspended in large volumes of PBS/PTU or HPL-containing PBS/PTU, kept in suspension and individual haemocytes allowed to attach on a glass surface. Haemocytes were fixed and actin-cytoskeleton visualised with FITC-conjugated phalloidin. (A) Haemocytes from haemolymph isolated in PBS/PTU. Note the evenly spread actin-cytoskeleton with a gap of staining over the nucleus. (B) Haemocytes from lectin-treated haemolymph. Note the reduced spreading and the dotted phalloidin-staining over the cytoplasm and around the nucleus.

4. Discussion

Our observations suggest that oligomeric lectins mediate uptake reactions on the cell surface by rearranging glycoprotein receptors and in the process destabilising actin-cytoskeleton. Since immobilised lectins also mediate spreading, and soluble lectins cause detachment, it is possible that the functional role of lectins is not limited to signal-transduction. Instead, our observations imply that some of the driving forces involved in these processes are lectin-mediated and may occur upstream of signalling events (Schmidt and Theopold, in press). Firstly, HPL can cluster glycoprotein receptors on the cell surface (Fig. 1). This is reminiscent of receptor-specific antibodies that are able to cluster and internalise receptors on the cell surface (Bunnell et al., 2002; Grow et al., 1999; Issafras et al., 2002). Secondly, HPL is able to mediate uptake reactions (Figs. 2 and 4), which are different from constitutive macropinocytosis (Johannes and Lamaze, 2002). Lectins or other oligomeric adhesion molecules have also been implicated in uptake reactions during lectinophagocytosis (Ofek et al., 1995) and receptor-internalisation reactions (Cochran et al., 2001; Conway et al., 1994; Muro et al., 2003), but the exact role of actin in these processes is not known. Since cytochalasin D treatment interferes with lectin-mediated vesicle-formation to a much lesser extent than with adhesion (Fig. 6) and phagocytosis (Vilcinskas et al., 1997a, b), we suspect that lectin-mediated uptake reactions involve lateral receptor cross-linking (Muro et al., 2003), whereas adhesion and phagocytosis reactions require actin-mediated receptor-stabilisation to allow contact to external binding sites. Thirdly, HPL-mediated uptake reactions affect F-actin (Fig. 6), possibly as a consequence of lectin-mediated membrane curvature (Bettache et al., 2003) and associated receptor rearrangements. As a result of the breakdown of F-actin, vesicle transport and membrane-recycling on the cell surface may become interrupted (Shupliakov et al., 2002). Taken together, these observations suggest that oligomeric lectins may be involved in extracellular receptor rearrangements that drive uptake reactions.

That raises the question, what is the function of F-actin in these processes? Given that F-actin appears to be less important for lectin-mediated vesicle formation than for adhesion and phagocytosis, one of the possibilities is that adhesive processes require receptor-stabilisation to occur. If receptors and oligomeric adhesion molecules are engaged in both adhesion and uptake reactions, the latter are probably favoured, given that membrane-receptors are already arranged in a two-dimensional membrane, whereas attachments to external binding sites involve interactions in three dimensions. In the presence of cell-derived or externally added oligomeric adhesion molecules, membrane-attached glycoproteins are therefore more likely to cluster and become internalised than to attach to external binding sites (Fig. 7). In this context it is possible that one of the functional roles of receptor-anchorage to the actin-cytoskeleton is to enhance adhesion over uptake reactions by preventing or delaying receptor-clustering and uptake reactions. Conversely, the observed destabilisation of actin-cytoskeleton by some lectins may be a consequence of lectin–receptor complexes that can create the configurational energy to overcome receptor-anchorage thereby dislocating existing connections to the actin-cytoskeleton. This is compatible with the notion that regulation of actin-cytoskeleton is important in mediating cell shape changes (Adams,

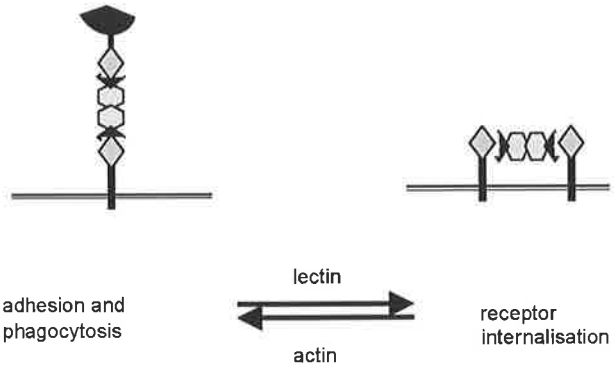

Fig. 7. Depiction of a dynamic equilibrium of lectin interactions on the cell surface. Glycoprotein receptors can either make contacts to external binding sites (adhesion or phagocytosis), or cause lateral cross-linking on the cell surface leading to receptor-internalisation (uptake reactions). Since some lectins with counter-adhesion properties internalise receptors from the cell surface and in the process depolymerise actin-cytoskeleton, lectin-mediated uptake will eventually cause depletion of cell surface receptors (immune suppression), as recycling of membrane-vesicles to the periphery requires actin-fibres. Conversely, strengthening of F-actin or other cytoplasmic protein scaffolds that stabilise cell surface receptors will shift the balance towards adhesive processes, such as spreading and phagocytosis.

1995; Etienne-Manneville and Hall, 2002; Rogers et al., 2003), but implies a role of actin-cytoskeleton in stabilising receptors against lectin-mediated internalisation rather than being a motor protein.

Since some cell surface receptors are able to interact both by cross-linking with lateral receptors and by linking with extracellular binding sites, the two reactions form a dynamic equilibrium. When receptor orientation on the cell membrane is stabilised, attachments to extracellular sites is more likely, while free lateral movements of receptors favour receptor cross-linking and internalisation. Stabilisation of receptor orientation can be achieved by receptor attachment to the intracellular actin cytoskeleton and by receptor–substrate linkages. Destabilisation occurs when receptor anchorage to the actin cytoskeleton is disrupted. In addition to the action of molecules such as cytocalasin D, oligomeric adhesion molecules with strong cross-linking abilities may be able to dislocates receptor-anchorage leading to receptor-internalisation and detachment from substrate, a feature of counter-adhesion molecules (Bornstein, 2001) and immune suppressors (Asgari et al., 1997).

Likewise, the observation that spread cells lose phagocytic ability in the presence of low concentrations of cytochalasin D (Vilcinskas et al., 1997) before detaching (Fig. 5) is in agreement with this model, given that receptor-stabilisation at focal adhesion points is enhanced by receptor linkages to external binding sites in addition to cytoplasmic actin-cytoskeleton anchorage.

This model also predicts the behaviour of polarised cells, where impairment of actin-containing microfilaments by cytochalasin D selectively inhibits the capacity of cells to endocytose membrane-bound and fluid-phase markers applied to the apical surface, without affecting endocytosis from the basolateral surface (Gottlieb et al., 1993). Following impairment of F-actin, receptor orientation on the apical surface is destabilised and receptors are internalised by lateral cross-linking. Since receptor-recycling on the cell surface requires intact actin fibres (Shupliakov et al., 2002), the outcome is receptor depletion and loss of endocytotic ability. In contrast, actin fibres are retained on the basolateral surface by receptor–substrate linkages and the cells endocytotic potential is maintained.

In summary, we propose adhesion and uptake reactions are part of a dynamic balance of interactions, where one of the roles of actin-cytoskeleton is to stabilise receptors on the cell surface to prevent clustering and internalisation in the presence of oligomeric lectins. In addition, linkages to external binding sites protect receptors from internalisation unless molecules with counter-adhesion properties can overcome the cytoplasmic receptor-anchorage and internalise receptors. In this model, the direct consequences of actin-cytoskeleton breakdown are receptor-internalisation and lack of membrane recycling at the adhesion site, causing detachment of spread cells from the substrate and cell-contraction into round or spindle-shaped cells.

Acknowledgements

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