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Testing the 'usurpation hypothesis' with the secondary hyperparasitoid *Lysibia nana*

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Content

Abstract

Parasitoids regulate the host during their development to increase parasitoid survival and fitness. Larvae of the primary parasitoid *Cotesia glomerata* feed primarily on the haemolyph and fat body of the host during their development. *C. glomerata* has evolved several mechanisms to keep its host (larvae in the genus *Pieris*, with its main host *P. brassicae*) in a suitable condition during the larval development. After egression, the larvae spin their cocoons close to the host. However, due to selective tissue destruction, the host does not die after parasitoid emergence, but remains close to the cocoons and sometimes even coils over the cocoons. The host caterpillar spins a silk layer to cover the cocoons, and might react aggressively when disturbed. The 'usurpation hypothesis' theory suggests that *C. glomerata* might be able to manipulate and use the behaviour of the surviving host after egression and pupation as a defence against predators and secondary hyperparasitoids. In this study, the 'usurpation hypothesis' has been tested with the secondary hyperparasitoid *Lysibia nana*. Different aspects of the host, being the presence, state and position of an attending caterpillar and the presence of a silk web covering the cocoons, were tested separately. The 'usurpation hypothesis' was tested on a number of different levels, being parasitoid survival, choices made by *L. nana* and *L. nana* foraging behaviour. As hypothesised, the presence, state and position of a caterpillar had no influence on parasitoid survival, or on *L. nana* foraging behaviour. Opposite to expectations, the presence of a silk web increased *C. glomerata* survival, and decreased *L. nana* survival. Furthermore, *L. nana* showed less oviposition behaviour on the silk-covered cocoons. However, *L. nana* did not prefer to stay on the bare cocoons compared to the covered cocoons. Potential olfactory cues emitted by the host or host by-products did not attract *L. nana*. The results of this study provide partial support for the 'usurpation hypothesis' in this multitrophic association: the presence of a silk web increased parasitoid survival, but the caterpillar itself did not increase parasitoid survival and not all caterpillars spin a silk web. Therefore the survival and presence of the host after parasitoid emergence did not necessarily lead to a higher parasitoid survival. Furthermore, since hyperparasitoids are much specialised and abandoning a potential host could decrease reproductive success significantly, which was also supported by the results, it is expected that the usurpation of host behaviour by parasitoids is much more aimed at protection from predators, instead of hyperparasitoids.

Introduction

Parasitic wasps lay their eggs inside or externally on a host and during their larval development the parasitoids feed on the haemolymph and other internal tissues of the host (Godfray 1994; Brodeur and Vet 1994; Harvey 2000). Afterwards, the larvae pupate and new parasitoids emerge from the cocoons (Godfray 1994; Harvey 2000). Parasitoids regulate the host during their development to increase parasitoid survival and fitness, and they influence the development, behaviour, physiology and morphology of the host (Vinson and Iwantsch 1980; Slansky 1986). These effects on the host are often due to factors such as polydnaviruses and virus-like particles injected into the host by the ovipositing female (Beckage and Gelman 2004), but can also be due to other parasitoid-derived products such as teratocytes (Dahlman 1991; Beckage and Gelman 2004).

Most species of parasitic wasps normally attack only a few host species (Godfray 1994; Harvey and Witjes 2005). They are much more specialised than arthropod predators, most of which are generalists and thus attack many different kinds of prey (Godfray 1994;

Harvey and Witjes 2005). Parasitoids exploit a highly nutritious resource, the host body. Furthermore, the costs of metabolic activity are small compared to actively foraging predators, because the parasitoid larvae are sessile (Slansky 1986). However, parasitoids depend on the resources provided by a single host, whereas predators need many prey items to reach maturity. A parasitoid must therefore make sure to optimally utilise this limited resource (Harvey et al. 2004b).

A primary parasitoid usually parasitizes the larvae of an herbivorous insect, whereas a hyperparasitoid parasitizes a primary parasitoid. Within the hyperparasitoids, a primary hyperparasitoid parasitizes the parasitoid larvae that are still inside their host and a secondary- or pseudohyperparasitoid parasitizes newly cocooned pre-pupae and pupae of a primary parasitoid (Harvey et al. 2003). At the end of their larval development the mature parasitoid larvae egress from the host and start to spin a cocoon to pupate within (primary parasitoids), or they pupate within the cocoon that the host had spun (primary (with a Lepidopteran secondary host) and secondary hyperparasitoids) (Harvey et al. 2003).

Two broadly different macroevolutionary host usage strategies have been described among parasitic wasps. Koinobiont parasitoids (including primary parasitoids and primary hyperparasitoids) develop in hosts that continue feeding, growing, and defending themselves during the early phases of parasitism, whereas idiobiont parasitoids (including secondary hyperparasitoids) develop in non-growing host stages, such as eggs or pupae (Askew and Shaw 1986; Harvey 2005; Harvey et al. 2006).

Because idiobiont hosts do not feed or grow, the host resources are effectively 'static' and the development of the parasitoid is dependent on the quality and amount of resources available at the time of oviposition (Mackauer and Sequeira 1993; Harvey 2005). Within idiobiont parasitoids, larger hosts are assumed to be of higher quality than small hosts, because they contain more resources for parasitoid development (Harvey et al. 2004b; Harvey 2005). However, as hosts age, they undergo dramatic morphological and physiological changes (sclerotization of the cuticle and differentiation of body parts into recognizable structures such as wings and antennae) and this inhibits the rate of consumption and digestion by the parasitoid larva. Therefore, older hosts will be less suitable for parasitoid development than younger hosts, even when these younger hosts are smaller (Harvey 2005; Harvey et al. 2006).

Koinobiont hosts continue feeding and growing and the relationship between host quality and parasitoid fitness is much more complicated than is the case with idiobionts (Mackauer and Sequeira 1993; Harvey 2005). Amongst koinobionts, the size of emerging adults often increases with host size at parasitism, but mortality in larger hosts can also be also higher (Harvey et al. 2004b). Consequently, overall parasitoid fitness may be a dome-shaped function of initial host size, which means that parasitoid fitness is optimized in hosts of intermediate size at parasitism (Harvey et al. 2004b). However, many koinobionts may attack hosts ranging in size from very small to large, almost full grown larval hosts (Harvey 2005).

In koinobiont parasitoids, the majority of the parasitoid larvae are known to consume most or all host tissues before they are able to pupate. These parasitoids require the entire host for their development and eventually kill the host through a depletion of nutrients, or through mechanical damage (Brodeur and Vet 1994; Harvey et al. 2000; Nakamatsu et al. 2006). However, some koinobiont parasitoid clades contain species that only feed on haemolymph and fat body (Brodeur and Vet 1994; Harvey et al. 2000; Nakamatsu et al. 2006). They do not consume the entire host, but the host remains alive after the egression of the parasitoid larvae (Brodeur and Vet 1994). The parasitoids emerge through the side of the host's abdominal segments, and they leave their second or third instar integuments as plugs in the egression holes to prevent the leaking of host haemolymph (Brodeur and Vet 1994; Nakamatsu et al.

2006). The larvae spin their cocoons immediately after emergence and pupate on or close to the host. The host remains alive, but does not feed and dies eventually after a few days (Brodeur and Vet 1994). Fat body and haemolymph feeding species are able to exploit a wider range of host sizes than tissue-feeders, because they are not obliged to consume the whole host (Harvey et al. 2000). Many tissue-feeding parasitoids may be forced to overeat when developing in very large hosts, or else are unable to escape from the host integument (Beckage and Templeton 1985; Harvey 1996).

Like other species in the higher Microgastrinae, larvae of the koinobiont parasitoid *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) feed primarily on haemolyph and fat body during their development. *C. glomerata* is a gregarious, primary parasitoid and attacks young larvae of several species of pierid butterflies, with its preferred host being *Pieris brassicae* (L.) (Lepidoptera: Pieridae) (Harvey et al. 2003; Harvey et al. 2006). *C. glomerata* has evolved several mechanisms to keep its host in a suitable condition during the larval development. These mechanisms include: selective tissue destruction (e.g. only consuming fat body and haemolymph) while they do not consume more than 50-60% of the host (Brodeur and Vet 1994; Harvey 2000); blocking host metamorphosis during the prepupal stage; temporary paralysis of the host to ensure easy cocoon spinning without abrupt host movements; and preventing host bleeding (which can inhibit pupation and increase risk of pathogen infection of the parasitic brood, but can also cause death of the host) by leaving plugs in the egression holes (Brodeur and Vet 1994; Nakamatsu et al. 2006). Furthermore, the growth of the host is often increased after parasitism due to an increase in food consumption and enhanced digestibility (Harvey 2000; Gu et al. 2003), although this effect depends on the number of parasitoids present (Harvey, 2000). After egression, the larvae spin their cocoons close to the host. The parasitoid cocoons are clustered, and they are fully detached from the immobile host. However, the host remains close to the cocoons and sometimes even coils over the cocoons (Brodeur and Vet 1994).

The percentage of the host that is consumed by the parasitoid larvae may be an important factor in determining the activity of the host after emergence of the parasitoids. Fat body is important in maintaining metabolic activity in insects (Wigglesworth 1984). Thus, the amount of fat body remaining in hosts after parasitoid egression may explain the activity level of the host. *C. glomerata* is known to consume about 70% of the host's fat body, although this depends on the brood size (J.A. Harvey, personal communication). This allows the host to survive for a few days, and to retain some level of physical activity, but the host remains effectively sessile and usually does not move far from the parasitoid cocoons. A recent theory suggests that the parasitoid might be able to manipulate and use the behaviour of the surviving host after egression and pupation in ways that increase the survival of the parasitoid brood (Brodeur and Vet 1994). This 'usurped' host behaviour – in the form of aggression towards any other insects that approach the cocoons brood - is apparently aimed at protection against natural enemies such as predators and hyperparasitoids (Brodeur and Vet 1994) and promotes parasitoid survival (Stamp 1981).

The 'usurpation hypothesis' of Brodeur and Vet (1994)

Brodeur and Vet (1994) studied host behaviour manipulation in *P. brassicae* caterpillars parasitized by *C. glomerata*. They found that following egression of the parasitoids from the host, the moribund caterpillar remained on the pupating parasitoids, spun a silk web over the parasitoid cocoons and responded aggressively when disturbed. This aggressive behaviour included head thrashing, biting and the regurgitation of fluids from the gut (Brodeur and Vet 1994). The silk web that covered the cocoons might protect the outer layer of cocoons against hyperparasitation by forming a physical barrier between the parasitoid and the cocoons. The silk web is expected to prevent hyperparasitoids from detecting the quality of the host,

because hyperparasitoids normally assess the developmental stage or physiological state of the host by direct antennal contact (Brodeur and Vet 1994; Tanaka and Ohsaki 2006).

Brodeur and Vet (1994) suggested that *C. glomerata* has the capacity to interfere with post-regression behaviour of the host. They argued that the wasp may have usurped some of the components of the host behaviour as a defence against predators and secondary hyperparasitoids. This is called the 'usurpation hypothesis'. The authors viewed the dying hosts as 'functional extensions' of the parasitoid. These 'functional extensions' contributed to the parasitoid survival during pupal development by protecting the pupae from natural enemies (Brodeur and Vet 1994).

Until now, two studies have been performed explicitly testing the 'usurpation hypothesis'. In the first study, using an insect predator, the 'usurpation hypothesis' has been supported (Kester and Jackson 1996). In this study it was found that *C. congregata* pupae that were attached to the host (the tobacco hornworm, *Manduca sexta L.*,) after emergence, suffered from lower mortality due to predation by the insect predator *Jalysus wickhami* than pupae that became dislodged after emergence from the host. The authors concluded that the lower mortality of attached pupae was most likely due to the direct defensive behaviour of the host, which included head-jerking, head twisting and 'spitting' towards the predator (Kester and Jackson 1996).

More recently, a study was conducted on the 'usurpation hypothesis' using a small hyperparasitoid wasp, *Trichomalopsis apanteroctena*. Tanaka and Ohsaki (2006) studied behavioural manipulation of host caterpillars by *C. glomerata* to construct defensive webs against hyperparasitism. Larvae of *C. glomerata* formed cocoon clusters after egression from the parasitized host caterpillar (*P. brassicae*). After the egression of parasitoids, the perforated host caterpillar lived for a short period and most of them constructed a silk web that covered the cocoon cluster. About 75% of the tested caterpillars spun a silk web, although only 25% spun one that totally covered the cocoons. Tanaka and Ohsaki examined whether these silk webs protected *C. glomerata* cocoons against the hyperparasitoid wasp *T. apanteroctena*. However, the rate of hyperparasitism did not differ between covered (with silk web) and bare (without silk web) cocoon clusters. As a result, silk webs did not protect the cocoons from hyperparasitoids in this experiment (Tanaka and Ohsaki 2006).

In this study, the 'usurpation hypothesis' is tested with the specialised secondary hyperparasitoid *Lysibia nana*. *L. nana* (Hymenoptera: Ichneumonidae) is a solitary, secondary idiobiont hyperparasitoid that attacks newly cocooned pre-pupae and pupae of several closely related gregarious endoparasitoids in the genus *Cotesia*, including *C. glomerata* (Schwarz and Shaw 2000; Harvey et al. 2003; Harvey et al. 2006). *L. nana* is an ectoparasitoid, which means that the eggs are laid externally on the body of the host, and not inside the host (Harvey 2000). Adult female wasps perforate the host cocoon with their ovipositor and inject permanently paralysing venom into the pre-pupa or pupa, followed by the laying of a single egg externally on the host. When the egg hatches, the larva perforates the host cuticle with its mandibles. It starts feeding on haemolymph, but as it grows it begins attacking all other tissues and eventually consumes the entire host (Harvey et al. 2006). The hyperparasitoid does not construct its own cocoon, but pupates within the cocoon previously constructed by its host (Harvey and Witjes 2005; Harvey et al. 2006). *L. nana* preferably parasitizes *C. glomerata* cocoons just after the cocoons have been made, and the parasitoids inside the cocoons are still pre-pupae (Harvey et al. 2003).

Contrary to earlier studies, this study focuses on a range of aspects within the 'usurpation hypothesis', and therefore gives a broad view on the influence of the presence of the host on parasitoid survival when a specialised secondary hyperparasitoid (*L. nana*) is involved. In this study, first the effect of the presence of the host caterpillar, the position of the host (on the cocoons or away from the cocoons) the state of the host (dead or alive), and

the presence of a silk web on the cocoons (spun by the host) on the survival of the parasitoid were tested. Furthermore, *L. nana* has been given the choice between parasitizing cocoon clusters with a different treatment (caterpillar present or absent and silk web present or absent) to test whether *L. nana* would prefer unattended cocoons towards attended cocoons and bare cocoons (without a silk web) towards covered cocoons (with a silk web). Third, the behaviour of *L. nana* during foraging and oviposition of *C. glomerata* cocoons under different circumstances (caterpillar present or absent and silk web present or absent) has been monitored.

Hypotheses

Hypothesis 1: When *L. nana* is given no choice, the presence of an attending caterpillar does not increase the chances of parasitoid survival.

Parasitic wasps normally attack only a few host species; they are much more specialised than most arthropod predators (Godfray 1994; Harvey and Witjes 2005). Any predator or hyperparasitoid that encounters a parasitoid cocoon cluster with an attending caterpillar has to decide whether or not to consume/parasitize the cocoon cluster or to search for alternative prey or hosts. If predators are repelled by the attending caterpillar or the silk web that was spun by the caterpillar to cover the cocoons, they can easily look for alternative prey (other types of insects). However, hyperparasitoids are normally quite specialised, and they do not have many alternative prey (Godfray 1994; Harvey and Witjes 2005). The hyperparasitoids rely on a few host species for their survival, and if they would abandon those hosts when a caterpillar would attend them, this would be a very costly decision. The hyperparasitoids depend on the host for their reproduction, and abandoning potential hosts will decrease their chance for reproduction.

Within the parasitoids group, *L. nana* is a much specialised secondary hyperparasitoid. It only attacks closely related wasps in the braconid subfamily Microgastrinae, which includes *C. glomerata* (Schwarz and Shaw 2000; Harvey and Witjes 2005). Therefore, *L. nana* is even more under intense selection to find and parasitize *C. glomerata* cocoons, whether a caterpillar is attending the cocoons or not. They will be very persistent in trying to parasitize *C. glomerata* cocoons. It is therefore expected that the presence of a caterpillar does not increase parasitoid survival, since *L. nana* will not abandon *C. glomerata* cocoons when a caterpillar is present, but will persist to try to parasitize the cocoons.

The presence and behaviour of the attending caterpillar can be broken down into different functional parts. These include the presence of the caterpillar, the state of the caterpillar (dead or alive), the position of the caterpillar (on the cocoons or off the cocoons) and the presence of a silk web that was spun by the caterpillar and covers the cocoons. These functional parts might separately influence parasitoid survival. However, since it is expected that the overall presence of a caterpillar will have no significant effect on parasitoid survival, it is also expected that just the presence, the state and the position of the caterpillar and the spinning of a silk web by the caterpillar will have no effect on the survival of *C. glomerata*.

> Sub-hypothesis 1a: The presence of an attending caterpillar does not increase parasitoid survival.
>
> Sub-hypothesis 1b: The state of an attending caterpillar (dead or alive) does not influence parasitoid survival

Sub-hypothesis 1c: The position of an attending caterpillar (on the cocoons or off the cocoons) does not influence parasitoid survival

Sub-hypothesis 1d: The presence of a silk layer, covering the parasitoid cocoons, does not increase parasitoid survival

Hypothesis 2: When given a choice, *L. nana* will prefer to parasitize *C. glomerata* cocoons that are not attended by a caterpillar compared to cocoons that are attended by a caterpillar.

L. nana has to parasitize *C. glomerata* cocoons, whether they are attended by a caterpillar or not, to make sure not to decrease reproductive success (see hypothesis 1).
However, when the secondary hyperparasitoid is given the choice between an attended cocoon cluster and a cluster that is not attended by a caterpillar, it will choose the easiest host. In this case, *L. nana* will parasitize the cocoons without an attending caterpillar, and it will leave the attended cocoons.
This preference for unattended cocoons will present itself in three ways:

Sub-hypothesis 2a: *L. nana* will be located on (or close to) the unattended cocoons more than on (or close to) the attended cocoons.

Sub-hypothesis 2b: Parasitoid survival of the cocoons that are attended by a caterpillar will be higher than parasitoid survival of the unattended cocoons.

Sub-hypothesis 2c: *L. nana* behaviour near an attended cocoon cluster is more disturbed than the behaviour near an unattended cocoon cluster. This more disturbed behaviour includes more walking away from the cocoons and less quickly and overall for a shorter period oviposition than near unattended cocoons.

Hypothesis 3: When given a choice, *L. nana* will prefer to parasitize *C. glomerata* cocoons that are not covered by a silk web compared to cocoons that are covered by a silk web.

As in hypothesis 2, it is expected that *L. nana* will choose the easiest host to parasitize. When given the choice between a bare (silk web absent) cocoon cluster and a covered (silk web present) cluster, *L. nana* will probably choose the bare cluster. This will present itself in the following three ways:

Sub-hypothesis 3a: *L. nana* will be located more towards the bare cocoons than towards the covered cocoons.

Sub-hypothesis 3b: *C. glomerata* survival of the cocoons that are covered by a silk web will be higher than *C. glomerata* survival of the bare cocoons.

Sub-hypothesis 3c: *L. nana* behaviour near a covered cocoon cluster is more disturbed than the behaviour near a bare cocoon cluster. As in hypothesis 2c, this more disturbed behaviour includes more walking away from the cocoons and less quickly and overall for a shorter period oviposition than near bare cocoons.

Hypothesis 4: When given a choice, *L. nana* will prefer to parasitize *C. glomerata* cocoons where no caterpillar and no silk web are present compared to cocoons where both a caterpillar and a silk web are present.

If *L. nana* can choose between a bare and unattended cocoon cluster and a silk-covered cocoon cluster with an attending caterpillar, it will prefer the former. This will be shown in the same way as hypothesis 2a, 2b and 2c and 3a, 3b and 3c.

Hypothesis 5: After parasitoid egression, the attending caterpillar survives long enough to allow the brood of *C. glomerata* to complete their pupal development and emerge as adult wasps.

In a study by Harvey et al. (2006), in hosts parasitized within the first 60 h after pupation secondary hyperparasitoid survival exceeded 80%, but the survival decreased afterwards. From 60 h to 84 h, the survival of *L. nana* decreased dramatically, and in hosts of more than 108 h old, the survival of the secondary hyperparasitoid was 0% (Harvey et al. 2006). Therefore, for an attending caterpillar to effectively protect the cocoon brood from enemies, it should live long enough to cover the cocoons' vulnerable period that lasts at least 84 h (3.5 days). Preferably, the caterpillar should attend the cocoons for at least 108 hours (5 days). It is therefore expected that most attending caterpillars live for at least 5 days.

However, the larger the brood size that fed on the caterpillar, the more fat body will be consumed. As suggested in the introduction, the percentage of fat body is important in maintaining metabolic activity in insects (Wigglesworth 1984). Therefore, it is expected that the more fat body is consumed, as a result of a larger parasitoid brood, the less active the caterpillar and the shorter the survival of the caterpillar will be.

Material and methods

Insect species

Pieris brassicae L. (Lepidoptera: Pieridae) is a specialist herbivore that feeds exclusively on plants that produce inducible glycoside toxins (glucosinolates) (Renwick and Lopez 1999; Sznajder and Harvey 2003). Larvae of *P. brassicae* feed on cultivated species such as cabbage (*Brassica oleracea* L) but also on wild species such as the black mustard (*Brassica nigra* L) (Harvey et al. 2003). On cultivated species they can become pests (Harvey et al. 2003). During early development, the larvae of *P. brassicae* are gregarious, whereas later instars disperse and may feed on other individual plants (Sznajder and Harvey 2003). An instar is a larval stadium, in which L1 is the youngest larva (just after the egg stage) and L5 is the oldest instar, just before pupation. *P. brassicae* larvae are able to protect themselves from parasitism by *C. glomerata* by encapsulating the parasitoids eggs or young larvae. However, the more parasitoid eggs that are deposited in the host, the less the defence reaction occurs (Gu et al. 2003).

Cotesia glomerata L. (Hymenoptera: Braconidae) is a host-specific gregarious endoparasitoid that attacks young larvae of several species of pierid butterflies, with *Pieris brassicae* L. (Lepidoptera: Pieridae) being its preferred host (Harvey et al. 2003). Females deposit between 10 and 40 eggs in the haemocoel of first-third instar (L1-L3) hosts, which continue feeding and growing until the final (fifth) instar (Brodeur and Vet 1994; Harvey et al. 2004a). The parasitoids feed on the host tissue (only fat body and haemolymph) and at the end of their larval development (during the host's final instar) the parasitoids egress together by forcing their way through the side of the host's abdominal segments (Brodeur and Vet 1994; Harvey et al. 2004a). Each parasitoid larva immediately begins spinning a cocoon and pupates (Brodeur and Vet 1994). The host dies within a few days (Harvey et al. 2004a). *C. glomerata* has a high fecundity and females have large egg loads at eclosion (Gu et al. 2003),

although at emergence only a small fraction of their eggs are fully mature and ready to be laid (Jervis et al. 2001; Harvey et al. 2003). They continue oögenesis during their life (Gu et al. 2003). *C. glomerata* produce small, yolk-poor eggs (Jervis et al. 2001; Harvey et al. 2003; Harvey and Witjes 2005). The development time of *C. glomerata* at 22 ± 2°C is between 18 and 20 days (J.A. Harvey, personal communication).

Lysibia nana Gravenhorst (Hymenoptera: Ichneumonidae) is a solitary secondary hyperparasitoid of newly cocooned pre-pupae and pupae in the braconid subfamily *Microgastrinae*, including *C. glomerata* (Schwarz and Shaw 2000; Harvey et al. 2003). Adult female wasps perforate the host cocoon with their ovipositor and inject permanently paralysing venom into the prepupa or pupa, followed by the laying of a single egg externally on the host. The paralysing venom might also increase the availability of host nutrients through the release of nutrients from host cells (Vinson and Iwantsch 1980). When the egg hatches, the larva perforates the host cuticle with its mandibles. It starts feeding on haemolymph, but as it grows it begins attacking all other tissues and eventually consumes the entire host (Harvey et al. 2006). The development time of *L. nana* at 22 ± 2°C is about 13 days (J.A. Harvey, personal communication). Females of *L. nana* have no fully mature eggs at eclosion (Harvey 2007). The eggs are matured in the days after eclosion. *L. nana* has a lower potential fecundity than *C. glomerata*, because female *L. nana* produce larger, yolk-rich eggs that contain a lot of proteins necessary for completion of the oögenesis, whereas *C. glomerata* produce smaller, yolk-poor eggs (Jervis et al. 2001; Harvey et al. 2003; Harvey and Witjes 2005). Under laboratory conditions, *L. nana* females will normally parasitize all cocoons in a single *C. glomerata* brood, unless they are disturbed during oviposition (Harvey et al. 2004a; Harvey and Witjes 2005). Larger females will produce more offspring than smaller females (Harvey 2007).

Insect cultures

All insects were reared at 22 ± 2°C under a 16:8h L:D regime. Cultures of *C. glomerata* and *P. brassicae* were obtained from insects maintained at Wageningen University (WUR), The Netherlands. *P. brassicae* larvae were reared on *Brassica oleracea var. Cyrus* (Brussels sprouts), on which the species has been maintained for several years at WUR. *C. glomerata* was reared according to the protocol described in Harvey (2000).

The *L. nana* culture originated from insects that emerged from cocoons of the parasitoid *C. glomerata* that were collected in field plots adjacent to the Institute of Ecology, Heteren, The Netherlands. *L. nana* was reared according to the protocol described in Harvey et al. (2003).

Experiments

All experiments were performed at the Entomology department, Wageningen University.

First instar (L1) larvae of *P. brassicae* were parasitized by *C. glomerata* females by allowing the wasps to sting the host for at least 15 seconds, which allowed them to oviposit a full brood. Parasitized caterpillars were reared on *Brassica oleracea* plants in rearing cages (65 x 40 x 48 cm). The parasitized caterpillars were transferred to petridishes when it became clear that the parasitoids were almost ready to emerge, which was shown by the caterpillars moving to the top of the food plant and sitting very quietly. 24 hours after parasitoid egression and pupation, the caterpillars and parasitoid cocoons were ready to be used in the experiments.

The female secondary hyperparasitoids were kept at 10°C for about 20 minutes before they were used in the experiments, to make sure that they were easy to handle and could be

put in the arena (petridish) without problems. The female secondary hyperparasitoids that were used came from a mixed culture (male and female), so male and female wasps could have emerged from the cocoons. The secondary hyperparasitoids were fed with honey before the experiments. All experiments were performed in petridishes with a diameter of 14.5 cm.

Experiment 1: Parasitoid survival

In this experiment, the effect of the presence, the state (dead or alive) and the position (on the cocoons or off the cocoons) of the caterpillar and the presence of a silk web on parasitoid survival were tested. To test these effects, 10 different treatments were used (table 1). Each treatment was repeated 15 times.

Table 1: List with the ten treatments that were used to test the effect of the presence, state (dead or alive) and position (on the cocoons or off the cocoons) of the caterpillar and the presence of the silk web on parasitoid survival.

Treatment	Caterpillar	State caterpillar	Position caterpillar	Silk layer
1	present	living	on cocoons	present
2	present	living	on cocoons	absent
3	present	dead	on cocoons	present
4	present	dead	on cocoons	absent
5	present	living	off cocoons	present
6	present	living	off cocoons	absent
7	present	dead	off cocoons	present
8	present	dead	off cocoons	absent
9	absent	n.a.	n.a.	present
10	absent	n.a.	n.a.	absent

First, it was analysed which aspects influenced the number of *C. glomerata* offspring, *L. nana* offspring and mortality when a caterpillar was present. These aspects included the state (living or dead) and position (dead or alive) of the caterpillar, and the presence of a silk web (table 1; treatment 1-8). Second, it was analysed if there was a difference in number of *C. glomerata* and *L. nana* offspring and mortality between attended and unattended cocoons (table 1; comparing treatment 1-8 with 9-10). Third, the effect of a silk web on (hyper)parasitoid survival was analysed by comparing covered and bare cocoons (table 1; treatment 9-10).

Caterpillars were taken off the cocoon clusters if necessary (caterpillar = absent). Caterpillars were killed if necessary (state = dead) by freezing them for 30-60 minutes, and these caterpillars were weighed before they were frozen. The silk layer was carefully peeled

off if necessary (silk layer = absent), but also cocoon clusters that were not covered by a silk web were used for the 'silk layer absent' treatments. It was carefully documented whether a silk layer was peeled off, or was not present in the first place, to test whether the peeling of silk had an influence on mortality.

After the treatment was applied, a single 5-10-day-old female secondary hyperparasitoid (*L. nana*) was released in the arena and was allowed to hyperparasitize the cocoons for 6 hours. After 6 hours, the secondary hyperparasitoid was removed from the arena and placed in new rearing cages in culture to ensure that they were used only once. Larvae of *P. brassicae* were then removed from the petridishes and weighed.

The *C. glomerata* cocoons per brood were counted and left in the arenas until adult parasitoid or secondary hyperparasitoid egression. Following egression, all adult *C. glomerata* and *L. nana* wasps were counted, as well as cocoons in which after 20 days neither species emerged (= 'dead cocoon'). Within this last group, it was also determined whether inside the cocoon there was an adult *C. glomerata,* an adult *L. nana* or an unidentifiable parasitoid present.

Experiment 2: *L. nana* choice experiment

In this experiment, the secondary hyperparasitoid was given the choice between two treatments to study the preference for a certain cocoon cluster (attended by a caterpillar or unattended and/or covered by a silk web or bare). The treatments were applied as described in experiment 1 (table 1). Three different sub-experiments were performed. In the first one, from now on to be called 'choice A', the female secondary hyperparasitoid was given the choice between an attended, covered cocoon cluster (treatment 1, see table 1) and an unattended, bare cocoon cluster (treatment 10). In the second one, 'choice B', the female secondary hyperparasitoid was given the choice between an attended, covered cluster (treatment 1) and an unattended, covered cocoon cluster (treatment 9). In the third one, 'choice C', the female secondary hyperparasitoid was given the choice between an unattended, covered cluster (treatment 9) and an unattended, bare cluster (treatment 10). Each sub-experiment was repeated 30 times.

In each sub-experiment, the two cocoon clusters with the different treatment were located on opposite sides of the arena (fig. 1). The arena was divided in 10 areas, with each area located on a fixed distance from the cocoon cluster. Areas 1 – 4 belonged to treatment α, in which area 1 was located on the cocoons, area 2 was located 0-2 cm from the cocoons, area 3 was located 2-4 cm from the cocoons and area 4 was located 4-6 cm from cocoons. Areas 5-8 belonged to treatment ß, and the areas were ordered the same as for treatment α, only now area 8 was located on the cocoons, and area 5 was located 4-6 cm from the cocoons. Area 9 was located more than 6 cm away from the cocoons of both treatments. Area 10 was the release location in the middle of the arena (fig. 1).

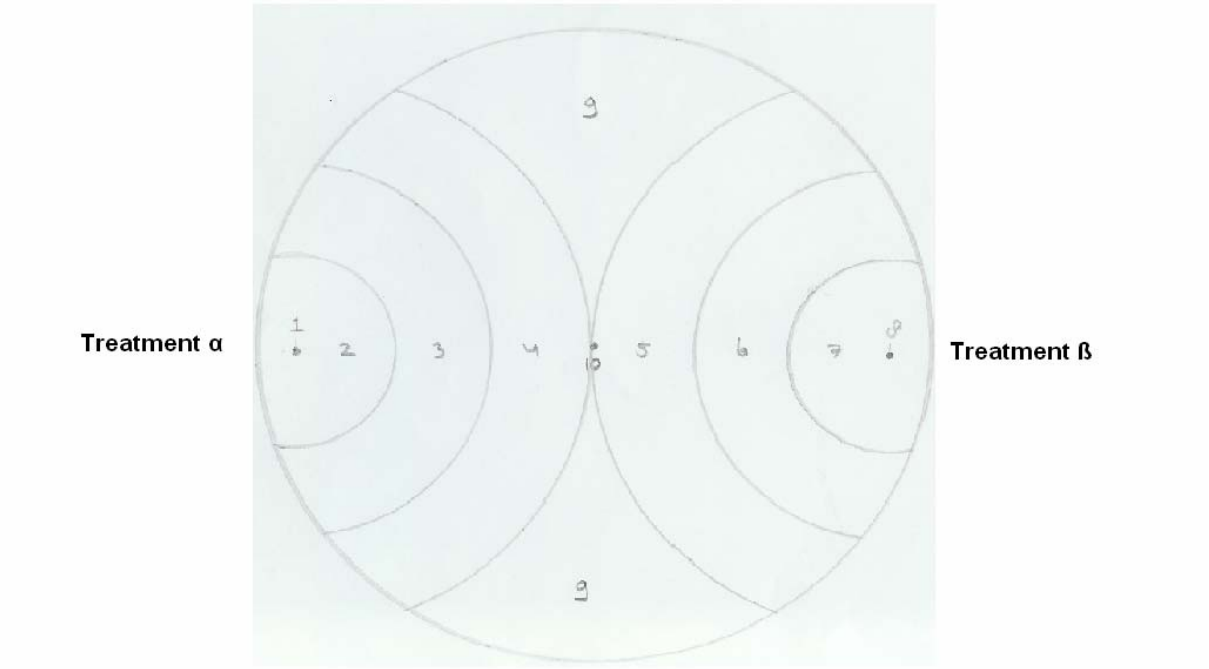

Figure 1: Representation of the arena used in experiment 2 which was divided into 10 areas. The two cocoon clusters were located on opposite sides of the arena. Every 30 minutes, it was recorded where the female *L. nana* was located.

The secondary hyperparasitoid could also walk on the lid of the petridish. If *L. nana* was walking on the lid of the petridish, the location was also determined. The number of the area on the lid was the same as in the petridish, only a 1 was added (so 0-2 cm from the cocoons of treatment α was area 12, 2-4 cm from the cocoons of treatment α was area 13 etc.)

The female secondary hyperparasitoid was released in the middle of the petridish and she was given 6 hours to parasitize the cocoons. The first choice (the cocoon cluster that was touched with the antennae first) was determined for every secondary hyperparasitoid. Furthermore, every 30 minutes it was recorded where she was located. The location of the secondary hyperparasitoid was determined based on the location of the head. After 6 hours, the secondary hyperparasitoid was removed from the petridish and placed in new rearing cages in culture to ensure that they were used only once. The caterpillars were then removed from the petridishes and weighed. The cocoon clusters of both treatments in each replicate were put in separate petridishes. The number of *C. glomerata*, *L. nana* and 'dead cocoons' were determined as described in experiment 1.

Experiment 3: *L. nana* behaviour observation

In this experiment, the behaviour of the secondary hyperparasitoid during foraging was recorded for (at least) 60 minutes. This was done using observer software. Three different treatments were observed separately: treatment 1 (attended, covered cocoons), treatment 9 (unattended, covered cocoons) and treatment 10 (unattended, bare cocoons), which are the same treatments as used in the choice experiments. During the observation, different behavioural elements were distinguished (table 2). For the behavioural elements *walk*, *sit* and *preen* it was also recorded if the secondary hyperparasitoid was located on the cocoons, nearby the cocoons (0-4 cm from the cocoons) or far away from the cocoons (> 4 cm from the cocoons).

Table 2: List of behavioural elements displayed by the secondary hyperparasitoid that were distinguished during the observation experiment. For walking, sitting and preening it was also recorded whether this was done on the cocoons, nearby the cocoons or further away from the cocoons.

Behavioural element	Position	Explanation of behavioural element	Key
release		Default: start value	0
walk	on cocoons	Moving around on cocoons, without movement of antennae. Antennae are not touching cocoons.	i
	nearby cocoons	Moving around nearby cocoons without flying. Antennae are not touching cocoons.	y
	further away	Moving around further away from cocoons without flying	w
sit	on cocoons	Staying on one place on the cocoons without moving and without movement of antennae. Antennae are not touching cocoons.	8
	nearby cocoons	Staying in one place nearby cocoons without moving. Antennae are not touching cocoons.	6
	further away	Staying in one place further away from cocoons without moving	2
preen	on cocoons	Cleaning of body with legs and mouth while situated on the cocoons	k
	nearby cocoons	Cleaning of body with legs and mouth while situated nearby the cocoons	h
	further away	Cleaning of body with legs and mouth while situated further away from the cocoons	s
fly		Moving around in the air	c
arrestment		Touching cocoons or caterpillar with antennae while the antennae are moving fast. This can be done while sitting or walking, and while being on the cocoons or off the cocoons	v
oviposition		Egg laying through penetration of host cocoons with ovipositor	b
probing		Ovipositor pointed downwards, but not laying egg	n
headbanging caterpillar		Caterpillar swings its headcapsule back and forth, mostly towards the secondary hyperparasitoid *(event, not state variable)*	p
touching by caterpillar		Movement of caterpillar (not headbanging) that leads to physical contact with the secondary hyperparasitoid *(event, not state variable)*	o

The female *L. nana* was released in the arena and immediately after release the behavioural observation was started. From the point in time of *L. nana*'s first arrestment of the cocoons, the observation lasted for a minimum of 30 minutes, and a maximum of 60 minutes.
For each of the three observed treatments, this observation experiment was repeated 25 times.

Experiment 4: Caterpillar survival

From the moment of emergence of the parasitoid larvae from the caterpillar, the brood size and the survival (in days) of 20 caterpillars were recorded.

Statistical analysis

Chi-square tests were performed in Microsoft Excel 1997. Contrasts following ANOVA were carried out in Statistica version 7. All other statistical tests were performed in SPSS 12.0 for Windows.

Experiment 1
The difference in brood size between treatments was analysed using an ANOVA on the square-root transformed brood sizes. The effect of the silk removal on mortality was analysed using a Mann whitney-U-test. To show the effect of the different aspects (state, position and silk) of the presence of a caterpillar on parasitoid (*L. nana* and *C. glomerata*) survival, an ANOVA with the brood size as a covariate (ANCOVA) was used, since there was a clear positive relationship between the brood size and number of *L. nana* offspring and between the brood size and number of *C. glomerata* offspring (see results). To analyse the effect of the state and position of the caterpillar and the presence of silk on mortality, an ANOVA on the log transformed data was used. The effect of the presence or absence of a caterpillar on the number of *L. nana* and *C. glomerata* offspring and on mortality was analysed using a Contrast-test (treatment 1-8 vs 9-10). The influence of the presence or absence of a silk web on parasitoid offspring was analysed using an ANOVA.

Experiment 2
The first choice for one of the two cocoon clusters in the choice experiments was analysed using a Chi-square test. To analyse if in a choice situation *L. nana* was located near the unattended and/or bare cocoon clusters more than near the attended and/or covered clusters, a Wilcoxon Signed Rank Test was used. The difference in number of *L. nana* offspring between the two treatments in each choice experiment was analysed using a t-test.

Experiment 3
The difference in time to arrestment between the three treatments was analysed using an ANOVA on the square-root transformed data. To analyse whether there was a difference between the treatments in the number of replicates in which *L. nana* was able to oviposit, a Chi-square test was used. The difference in number of ovipositions was analysed using an ANOVA followed by a Tukey, and the difference in mean total oviposition time was analysed using a Kruskal Wallis followed by a Scheffe. The mean time per oviposition was analysed using an ANOVA on the double squared-root transformed data. To analyse the difference in walking away from the cocoons by *L. nana* between the treatments, a Chi-square test was performed on the number of replicates in which *L. nana* went far away from the cocoons and an ANOVA was done on the square-root transformed mean total time that was spend far away

from the cocoons. A Kruskal Wallis was used to analyse the difference in the number of times that *L. nana* went outside the circle that contained the cocoons between the treatments. The effect of the headbanging and touching of the caterpillar on *L. nana* behaviour was analysed using a paired t-test on the square-root transformed number of times that *L. nana* did change and did not change its behaviour after headbanging and touching.

Experiment 4
The effect of brood size on caterpillar survival was analysed using a linear regression.

Results

Experiment 1: Parasitoid survival

The mean brood size for all treatments was 22.49 ± 0.61 (SE). There was no difference in brood size between treatments ($F_{9,140}$ = 0.663; P = 0.742). Silk removal had no effect on mortality (Z = -1.426; P = 0.154).

Influence of the state and position of the caterpillar and the presence of the silk web on *L. nana* offspring, *C. glomerata* offspring and mortality
When a caterpillar was present, the presence of a silk web that covered the cocoons decreased the number of *L. nana* offspring (table 3; fig. 2). The other two factors, the state (living or dead) and the position (on or off the cocoons) of the caterpillar, had no influence on the number of *L. nana* offspring (table 3; fig. 2). The interactions between these three factors also had no influence on the number of emerging *L. nana* (table 3).

Table 3: Results of the ANCOVA on the effect of the state and position of the caterpillar, the presence of a silk web, and the interactions of these factors on the number of *L. nana* offspring. Brood size was used as a continuous variable

Source	**Sum of squares**	**df**	**Mean Square**	**F**	***P***
Brood size	542.373	1	542.373	10.690	0.001
State	67.935	1	67.935	1.339	0.250
Position	88.589	1	88.589	1.746	0.189
Silk	208.017	1	208.017	4.100	0.045
State * Position	25.573	1	25.573	0.504	0.479
State * Silk	47.048	1	47.048	0.927	0.338
Position * Silk	106.668	1	106.668	2.102	0.150
State * Position * Silk	52.037	1	52.037	1.026	0.313
Error	5631.893	111	50.738		

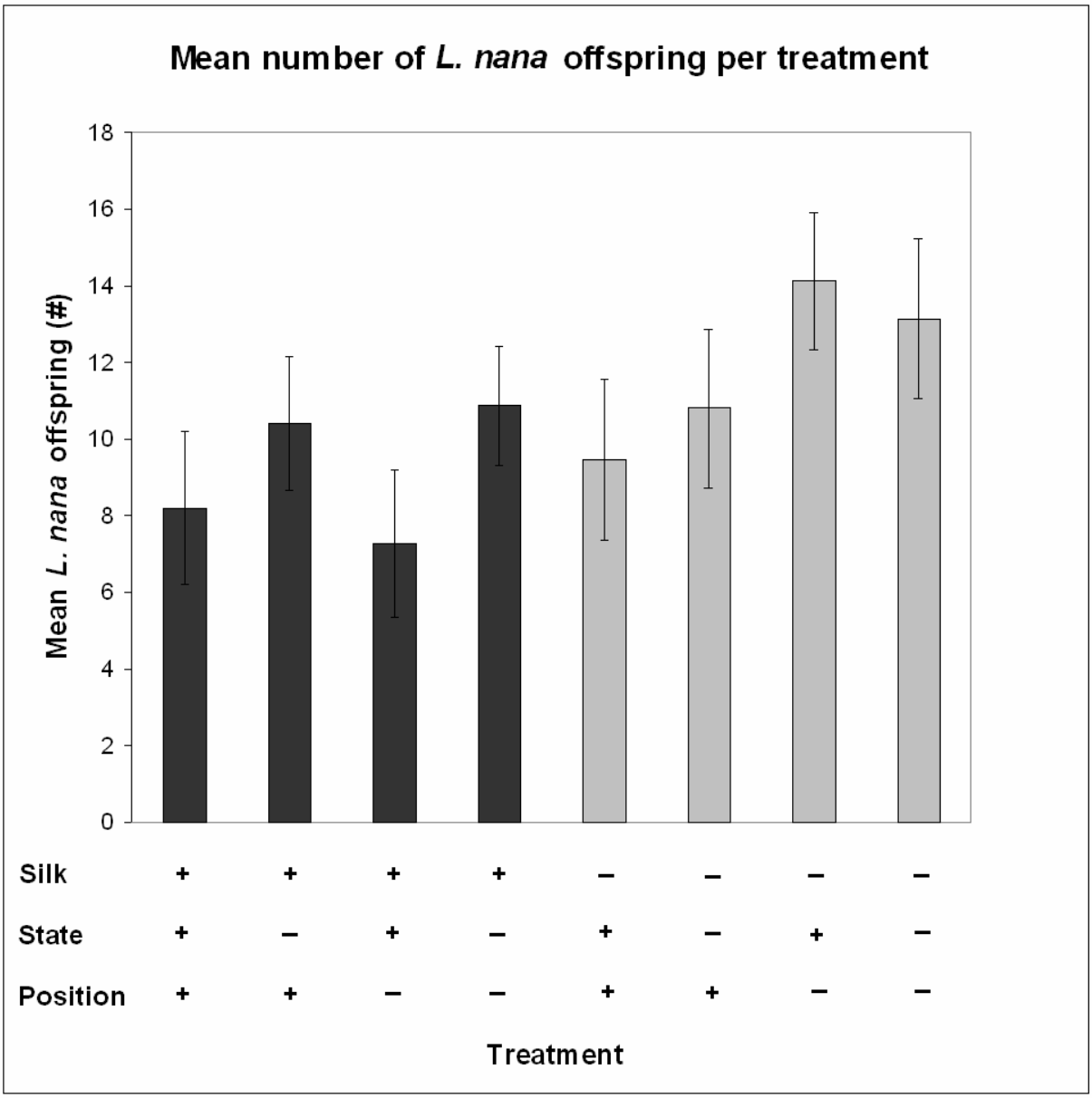

Figure 2: Mean number of *L. nana* offspring per treatment. Treatments consist of different aspects, which are silk present (silk +, dark grey bars), silk absent (silk –, light grey bars), living caterpillar (state +), dead caterpillar (state –), caterpillar on cocoons (position +) and caterpillar off cocoons (position –). Means are shown (+/- SE). Sample size is 15 per treatment.

In congruence, when a caterpillar was present the number of *C. glomerata* offspring was only influenced by the presence of a silk web that covered the cocoons. The presence of silk increased the number of *C. glomerata* (table 4; fig. 3). The state and the position of the caterpillar had no influence on the number of *C. glomerata* (table 4; fig. 3). The interaction terms of these factors also had no influence on *C. glomerata* offspring.

Table 4: Results of the ANCOVA on the effect of the state and position of the caterpillar, the presence of a silk web, and the interactions of these factors on the number of *C. glomerata* offspring. Brood size was used as a continuous variable

Source	**Sum of squares**	**df**	**Mean Square**	**F**	***P***
Brood size	3421.040	1	3421.040	67.426	0.000
State	67.935	1	67.935	1.339	0.250
Position	88.589	1	88.589	1.746	0.189
Silk	208.017	1	208.017	4.100	0.045
State * Position	25.573	1	25.573	0.504	0.479
State * Silk	47.048	1	47.048	0.927	0.338
Position * Silk	106.668	1	106.668	2.102	0.150
State * Position * Silk	52.037	1	52.037	1.026	0.313
Error	5631.893	111	50.738		

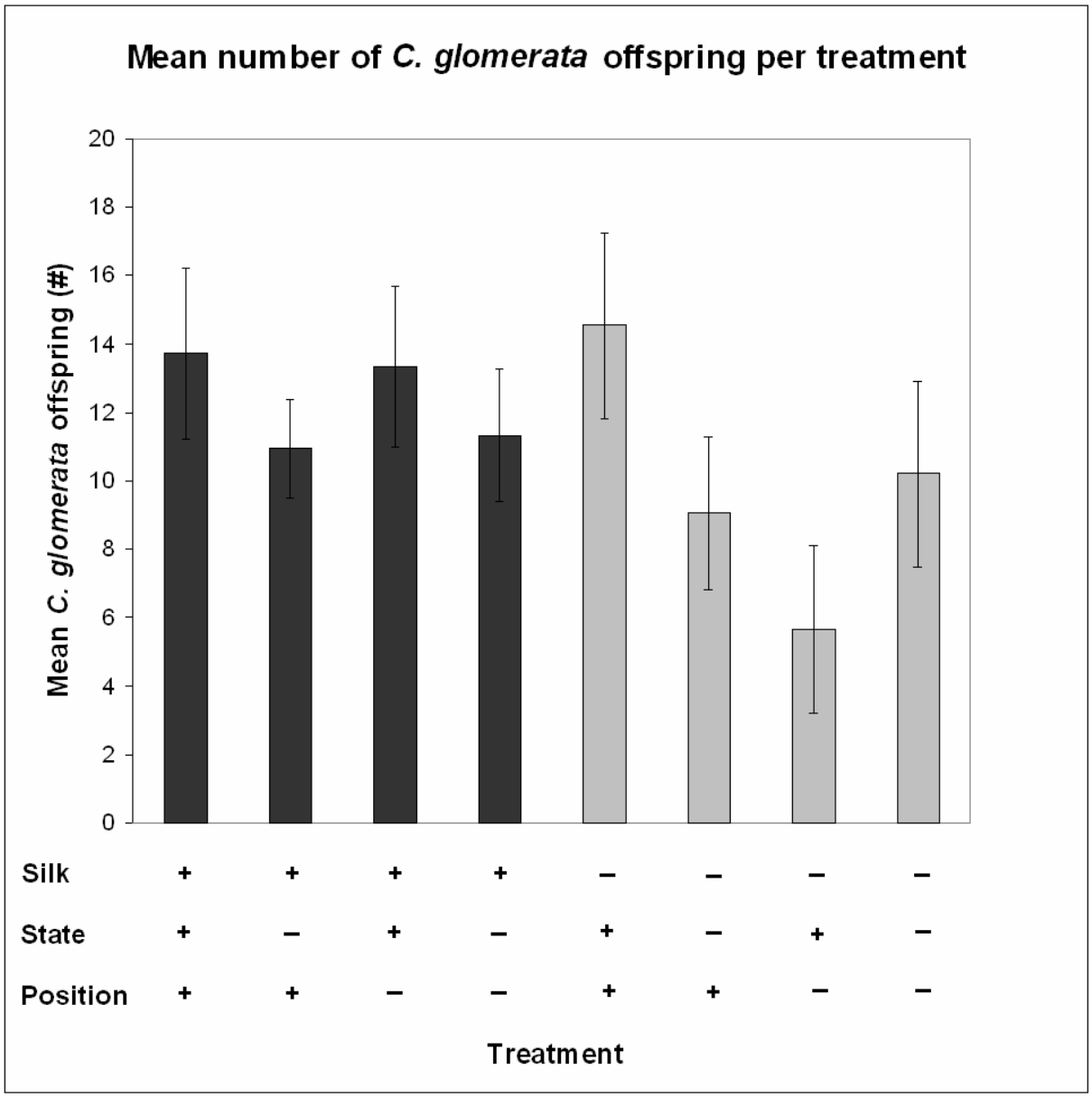

Figure 3: Mean number of *C. glomerata* offspring per treatment. Treatments consist of different aspects, which are silk present (silk +, dark grey bars), silk absent (silk –, light grey bars), living caterpillar (state +), dead caterpillar (state –), caterpillar on cocoons (position +) and caterpillar off cocoons (position –).Means are shown (+/- SE). Sample size is 15 per treatment.

There was no difference in the mortality (dead *C. glomerata* plus unidentifiable parasitoids) between covered and bare cocoons (table 5; fig. 4**)**, so the presence of a silk web did not influence mortality. There was also no difference in mortality between cocoon clusters attended by a living caterpillar and clusters attended by a dead caterpillar (table 5; fig. 4), so the state of a caterpillar had no influence on mortality. The position of a caterpillar (on or off the cocoons) also had no influence on the mortality (table 5; fig. 4). Furthermore, there were no significant interactions between the three factors (table 5).

Table 5: Results of the ANOVA on the effect of the state and position of the caterpillar, the presence of a silk web, and the interactions of these factors on the (log transformed) mortality.

Source	**Sum of squares**	**df**	**Mean Square**	**F**	***P***
State	0.016	1	0.016	0.041	0.840
Position	1.404	1	1.404	3.519	0.063
Silk	0.000	1	0.000	0.000	0.998
State * Position	0.151	1	0.151	0.379	0.539
State * Silk	0.034	1	0.034	0.085	0.771
Position * Silk	1.298	1	1.298	3.253	0.074
State * Position * Silk	0.014	1	0.014	0.034	0.854
Error	44.686	112	0.399		

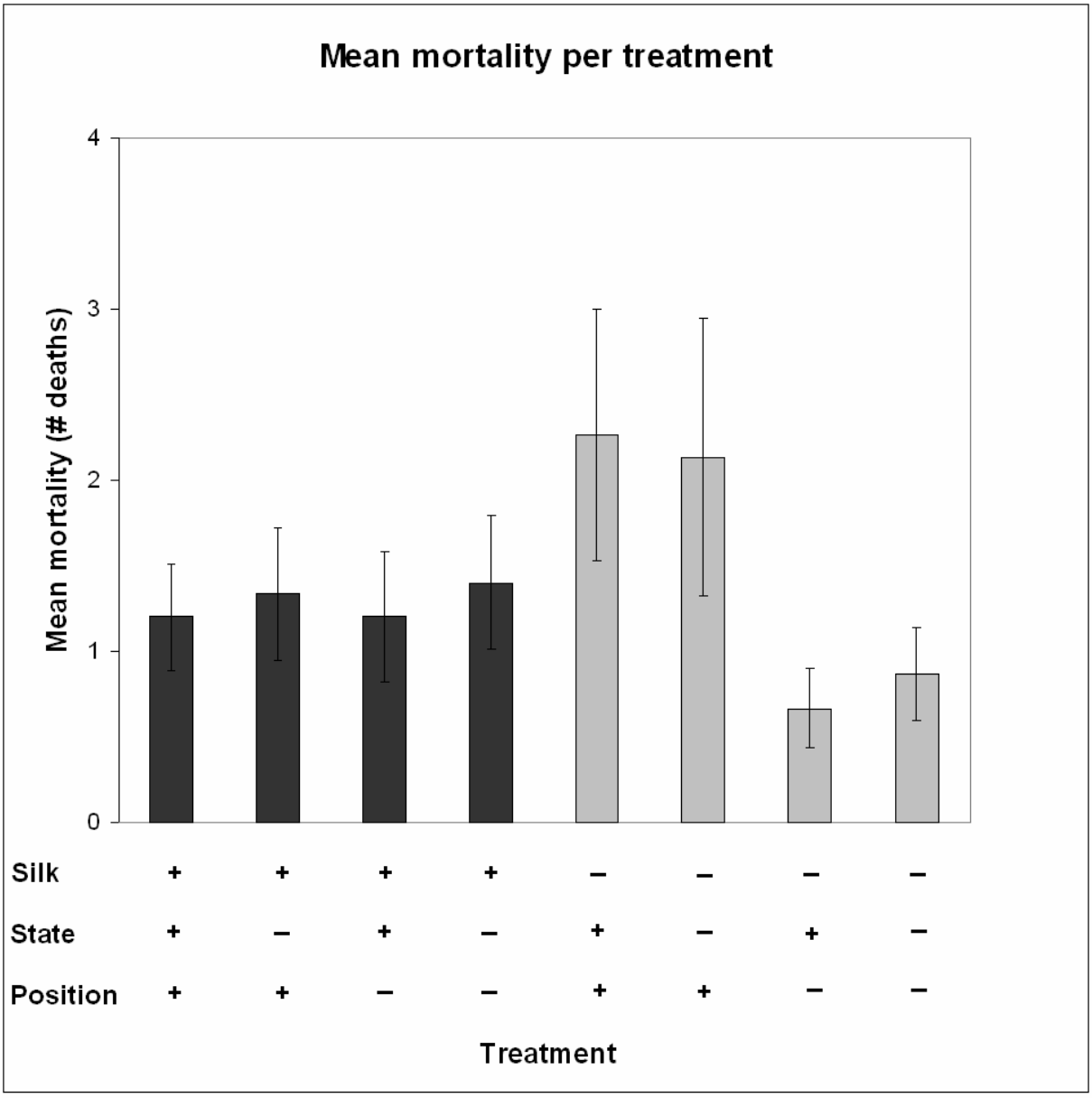

Figure 4: Mean mortality per treatment. Treatments consist of different aspects, which are silk present (silk +, dark grey bars), silk absent (silk –, light grey bars), living caterpillar (state +), dead caterpillar (state –), caterpillar on cocoons (position +) and caterpillar off cocoons (position –). Means are shown (+/- SE). Sample size is 15 per treatment.

Influence of an attending caterpillar on *L. nana* and *C. glomerata* offspring and mortality

Contrast analyses showed that there was no significant effect of the presence or absence of a caterpillar on the number of *L. nana* offspring ($F_{1,139} = 0.175$; $P = 0.676$) or on the number of *C. glomerata* offspring ($F_{1,139} = 0.892$; $P = 0.347$) (fig. 5). There was also no significant effect of the presence or absence of a caterpillar on mortality ($F_{1,139} = 0.006$; $P = 0.937$).

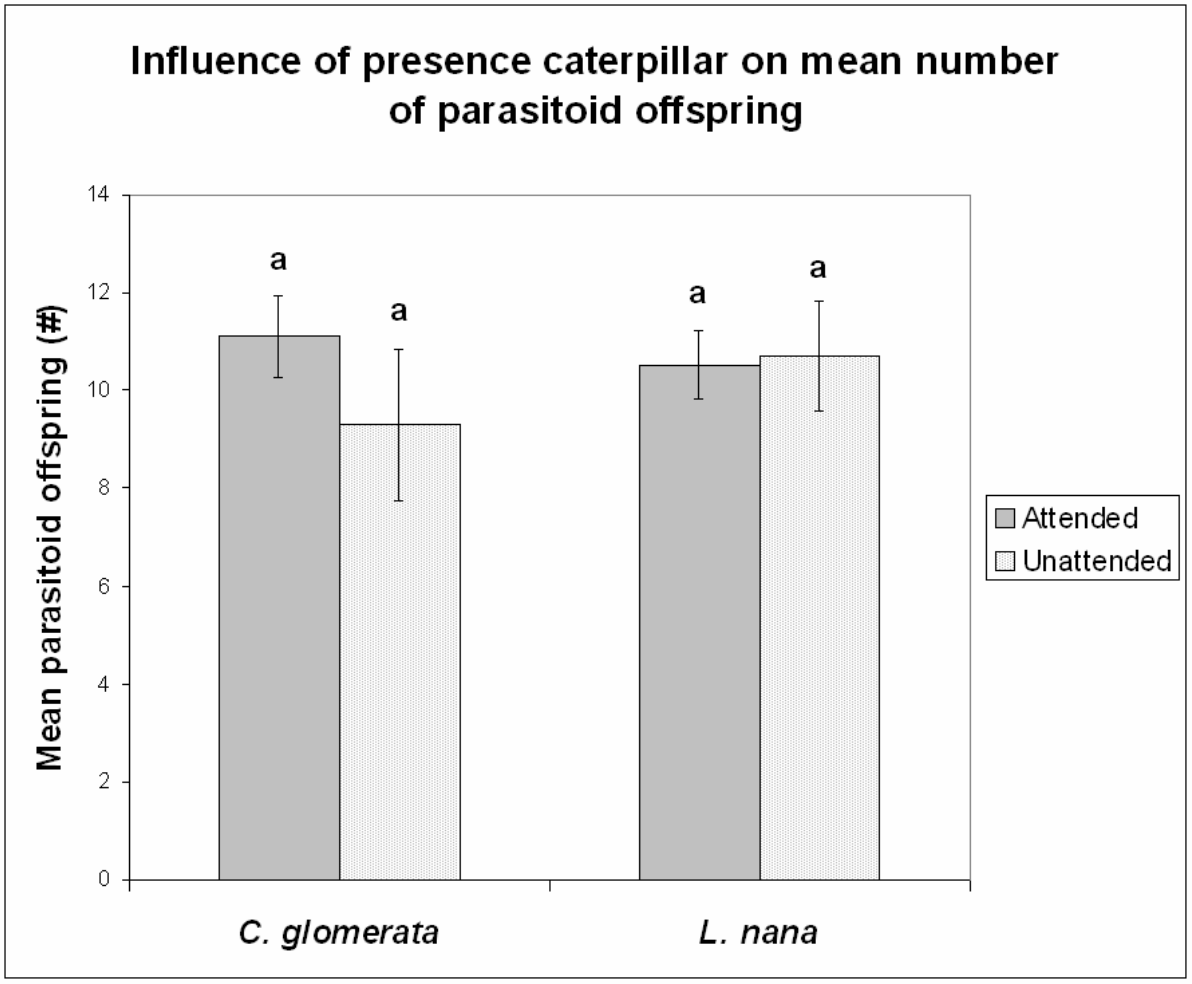

Figure 5: Influence of the presence (attended) or absence (unattended) of a caterpillar on the mean number of *C. glomerata* offspring and *L. nana* offspring. Means are shown (+/- SE). Within a species (*C. glomerata* or *L. nana*), bars with different letters differ significantly ($p < 0.05$; Contrast-test). Sample size for the unattended treatment = 120, sample size for the attended treatment = 30.

Influence of a covering silk web on *L. nana* and *C. glomerata* offspring

It was shown in the previous analyses that the presence of a silk web had a positive effect on the number of *C. glomerata* and a negative effect on the number of *L. nana* when a caterpillar was present, whereas the presence of the caterpillar itself had no effect. It was therefore decided to carry out another experiment in which only the effect of the presence of a silk web on the parasitoid survival was tested. This experiment consisted of two treatments, both without a caterpillar present, but one treatment did have a silk web that covered the cocoons, whereas the other one was bare (without a silk web).

The presence of a silk web had a negative effect on the number of *L. nana*. ($F_{1,27}$ = 10.563; $P < 0.01$). The average number of *L. nana* was 7.53 ± 1.29 with covered cocoon clusters, whereas it was 13.87 ± 1.46 with bare cluster (fig. 6)**.** The presence of a silk web had a positive effect on the number of *C. glomerata*. ($F_{1,27}$ = 9.969; $P < 0.01$). The average number of *C. glomerata* was 11.93 ± 2.54 with covered cocoon clusters, whereas it was 6.67 ± 1.56 with bare cluster (fig. 6).

Therefore, in absence of a caterpillar, the presence of a silk web had a positive influence on *C. glomerata* and a negative influence on *L. nana* offspring.

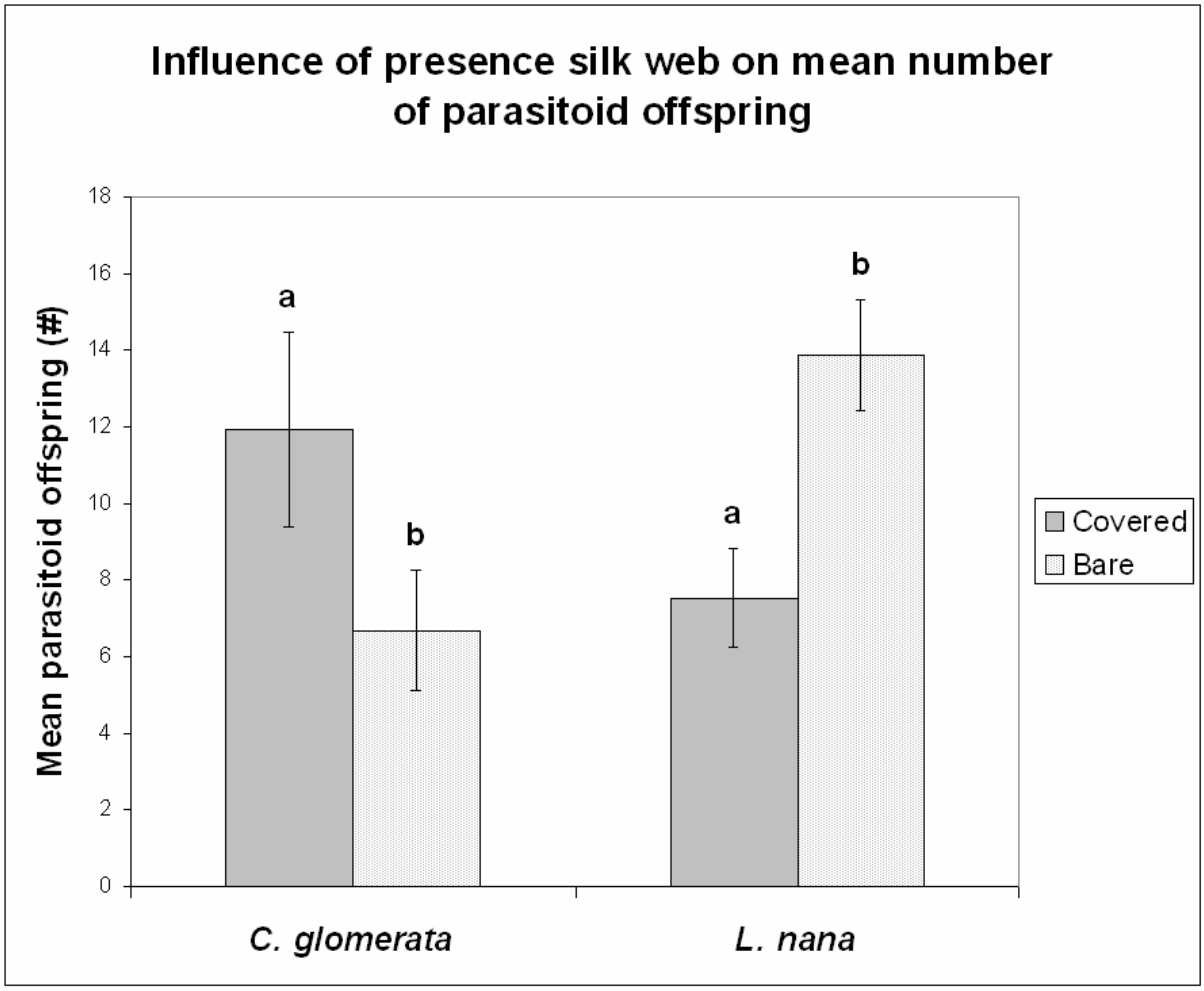

Figure 6: Influence of the presence (covered) or absence (bare) of silk web on the number of *C. glomerata* offspring and *L. nana* offspring. Means are shown (+/- SE). Within a species (*C. glomerata* or *L. nana*), bars with different letters differ significantly ($p < 0.05$; Contrast-test). Sample size for the covered treatment = 15, sample size for the bare treatment = 15.

Experiment 2:* L. nana *choice experiment

For the first choice, for the location of *L. nana* that was determined every 30 minutes for 6 hours, and for the number of *L. nana* offspring, all three choices ('choice A', 'choice B' and 'choice C') were analysed separately.

First choice

Choice A

When secondary hyperparasitoids had the choice between an attended, covered cocoon cluster and an unattended, bare cluster ('choice A') there was no difference in the cluster of first choice (Chi-square = 1.20; df = 1; P = 0.273): 60% (18 out of 30) went to the attended, covered cocoon cluster first, whereas 40% (12 out of 30) went to the unattended, bare cocoon cluster first (fig. 7).

Choice B

When *L. nana* had the choice between an attended and an unattended cocoon cluster (both covered by a silk web; 'choice B'), there was also no difference in the cluster of first choice (Chi-square = 3.33; df = 1; P = 0.067): 67% (20 out of 30) went to the attended cocoon cluster first, whereas 33% (10 out of 30) went to the unattended cluster first (fig. 7).

Choice C

When *L. nana* had the choice between a covered and a bare cocoon cluster (both unattended by a caterpillar; 'choice C'), again there was no difference in the cluster of first choice (Chi-square = 0.53; df = 1; P = 0.465): 57% (17 out of 30) went to the covered cocoon cluster first, whereas 43% (13 out of 30) went to the bare cluster first (fig. 7).

Although more secondary hyperparasitoids were going to the cocoon clusters where a caterpillar and/or a silk web were present first, this first preference for an attended and/or covered cluster was not significant (see fig. 7).

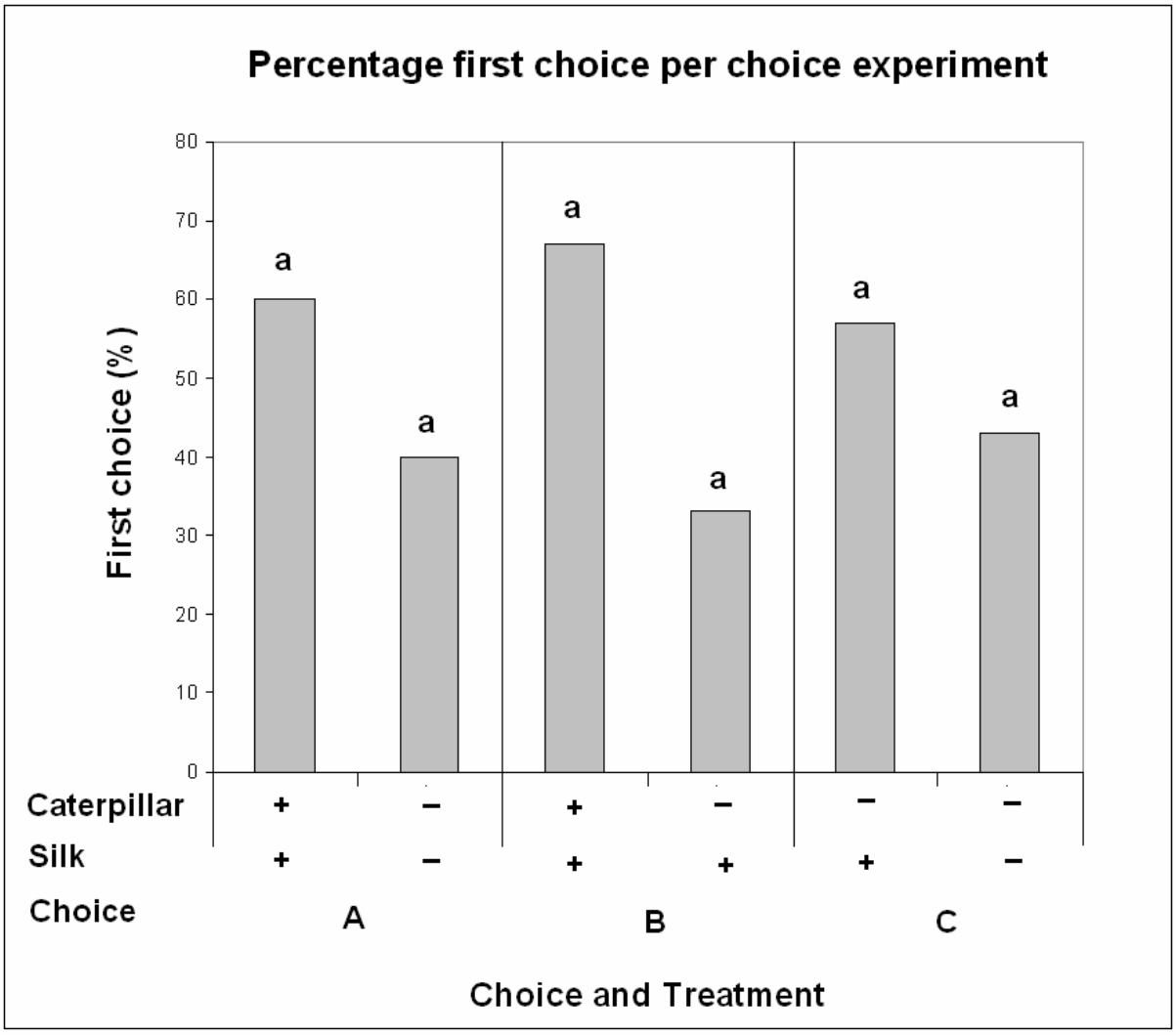

Figure 7: Percentage first choice per choice experiment. + represents present and – represents absent. Within a choice experiment ('Choice A', 'B' or 'C'), bars with different letters differ significantly ($p < 0.05$; Wilcoxon Signed Rank Test). Sample size per choice experiment =30.

Location determined every 30 minutes

To analyse further whether *L. nana* was located towards one of the two cocoon clusters in each choice experiment, the location of *L. nana* was grouped in three categories: 'touching cocoon cluster α' (location 1 and 2), 'touching cocoon cluster ß' (location 7 and 8) and 'Other' (all other locations). The replicates in which *L. nana* was on neither clusters for more than half of the time were deleted (so more than 3 hours in group 'Other').

Choice A

There was no difference in amount of time spend on (or very close to) the attended, covered cocoon cluster (4.92 ± 0.99 time units of 30 minutes) and on (or very close to) the unattended, bare cocoon cluster (6.54 ± 1.04 time units of 30 minutes) ($Z = -0.825$; $P = 0.409$).

Choice B

There was also no difference in amount of time spend on the attended, covered cocoon cluster (6.48 ± 1.12 time units of 30 minutes) and on the unattended, covered cocoon cluster (4.40 ± 1.12 time units of 30 minutes) ($Z = -1.042$; $P = 0.297$).

Choice C
Again, there was no difference in amount of time spend on the cocoon cluster between the two treatments, in this case an unattended, covered cluster (6.10 ± 1.02 time units of 30 minutes) and an unattended, bare cluster (5.48 ± 1.01 time units of 30 minutes) ($Z = -0.612$; $P = 0.540$).

Number of *L. nana* offspring
It was also analysed how many *L. nana* offspring emerged from each of the two cocoon clusters in each choice experiment, to test whether there was a difference in number of offspring when a cocoon cluster was attended or unattended, and covered with a silk web or bare. It was only analysed if there was a difference in number of *L. nana* offspring between the clusters when the secondary hyperparasitoid was really parasitizing the particular cluster (so 0-counts were deleted) because in the former analyses it was already analysed if certain clusters were preferred by the secondary hyperparasitoid. However, in all three choice experiments, there was no difference in the mean number of *L. nana* offspring between the two cocoon clusters ($t_{29} = -0.673$; $P = 0.507$ for 'Choice A'; $t_{19} = -0.915$; $P = 0.372$ for 'Choice B'; $t_{34} = -1.833$; $P = 0.076$ for 'Choice C').

Experiment 3: L. nana *behaviour observation*

In this observer experiment, the behaviour of *L. nana* and the behaviour of the caterpillar were recorded and analysed. Since the headbanging of the caterpillar (and the touching of *L. nana* by the caterpillar) was a quite striking feature, it was first analysed what the effect of this headbanging (and touching) was on *L. nana* behaviour.

Influence of behaviour of caterpillar on *L. nana* behaviour
Only if the behaviour of *L. nana* changed within 3 seconds after the headbanging or touching by the caterpillar, a change in behaviour was recorded. *L. nana* significantly more often did not change its behaviour after headbanging of the caterpillar or touching by the caterpillar than that it changed its behaviour following such event ($t_{24} = -3.651$; $P < 0.01$).

However, to be able to make conclusions about the effect of an attending caterpillar, or a covering silk web, the three different treatments must be compared on different variables such as time to first arrestment, different variables focussed on oviposition and different variables focussed on abandoning of the cocoons by *L. nana*. Therefore, the behaviour of *L. nana* during searching and oviposition of cocoons with different treatments was analysed on these variables. Since some female secondary hyperparasitoids found the cocoons almost immediately, whereas others took several tens of minutes, it was decided to test oviposition related behaviour and cocoon abandoning behaviour on the data during the first 30 minutes following first arrestment. In this way the results can be compared between replicates and treatments.

Time to arrestment
The mean time of *L. nana* to arrestment for the attended, covered cocoons was 568 ± 101 seconds, the average time to arrestment for unattended, covered cocoons was 737 ± 128 seconds and for unattended, bare cocoons it was 867 ± 181 seconds. This difference was not significant ($F_{2,72} = 0.505$; $P = 0.605$; fig. 8).

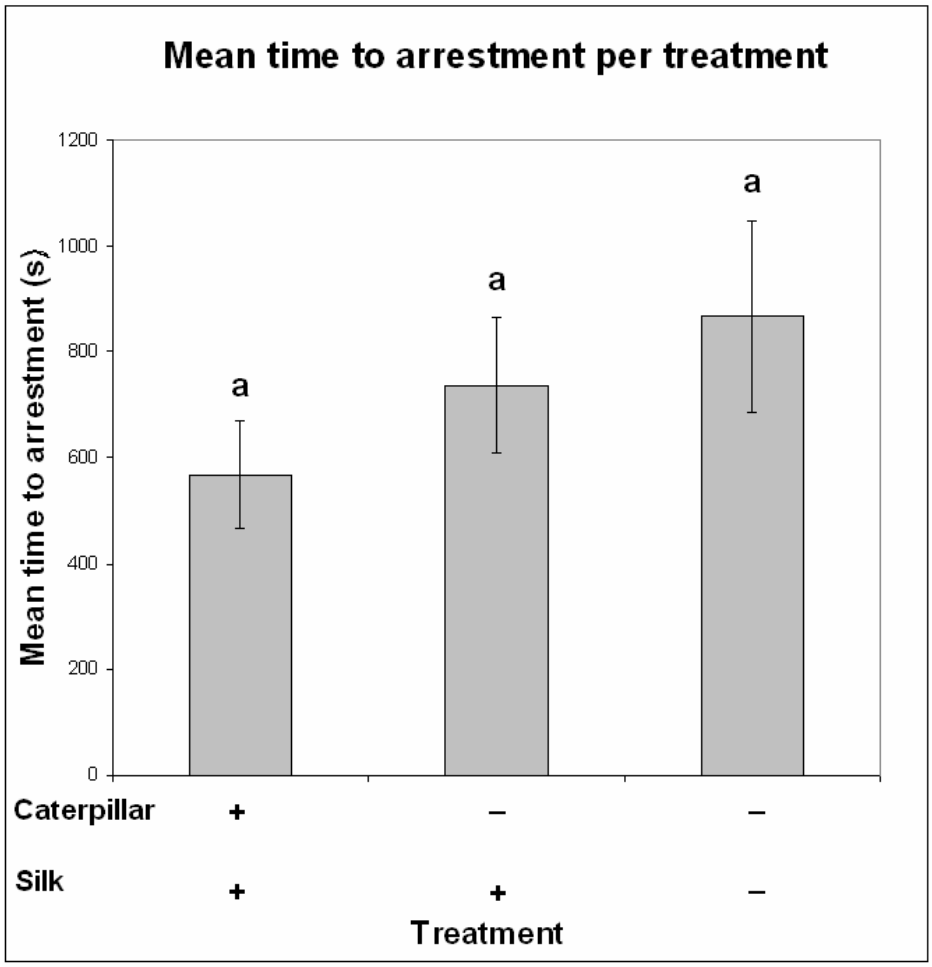

Figure 8: The mean time to arrestment per treatment. + represents present and – represents absent. Bars with different letters differ significantly ($p < 0.05$; ANOVA). Means are shown (+/- SE). Sample size is 25 per treatment.

Oviposition measurements

Did oviposition occur in the first 30 minutes after arrestment?

Ovipositions that lasted for less than 60 seconds were deleted from the analyses, since oviposition normally lasts between several minutes and 2 hours (Harvey et al. 2006). The number of replicates in which the secondary hyperparasitoid was able to oviposit in the first 30 minutes after arrestment was higher with the bare cocoons (23 out of the 25 replicates) than with both treatments of covered cocoons (8 and 9 out of the 25 replicates) (Chi-square = 22.607; df = 2; $P < 0.001$; fig. 9).

When oviposition occurred, how many times did it occur and how long in total did it last in the first 30 minutes after arrestment?

Of the replicates in which *L. nana* was able to oviposit, during the first 30 minutes after arrestment oviposition lasted for 234 ± 65 seconds on attended and covered cocoons, 619 ± 145 seconds on unattended but covered cocoons and 744 ± 66 seconds on unattended and bare cocoons. The mean total oviposition time on unattended, bare cocoons was higher than the mean total oviposition time on attended, covered cocoons ($F_{2,37} = 7.194$; $P < 0.01$; fig. 9).

The mean number of ovipositions was higher on the unattended, bare cocoons (2.04 ± 0.20 ovipositions) than on the unattended, covered cocoons (1.11 ± 0.11 ovipositions) (Chi-

square = 11.364; df = 2; $P < 0.01$). The mean number of ovipositions on the attended, covered cocoons was intermediate (1.25 ± 0.16 ovipositions).

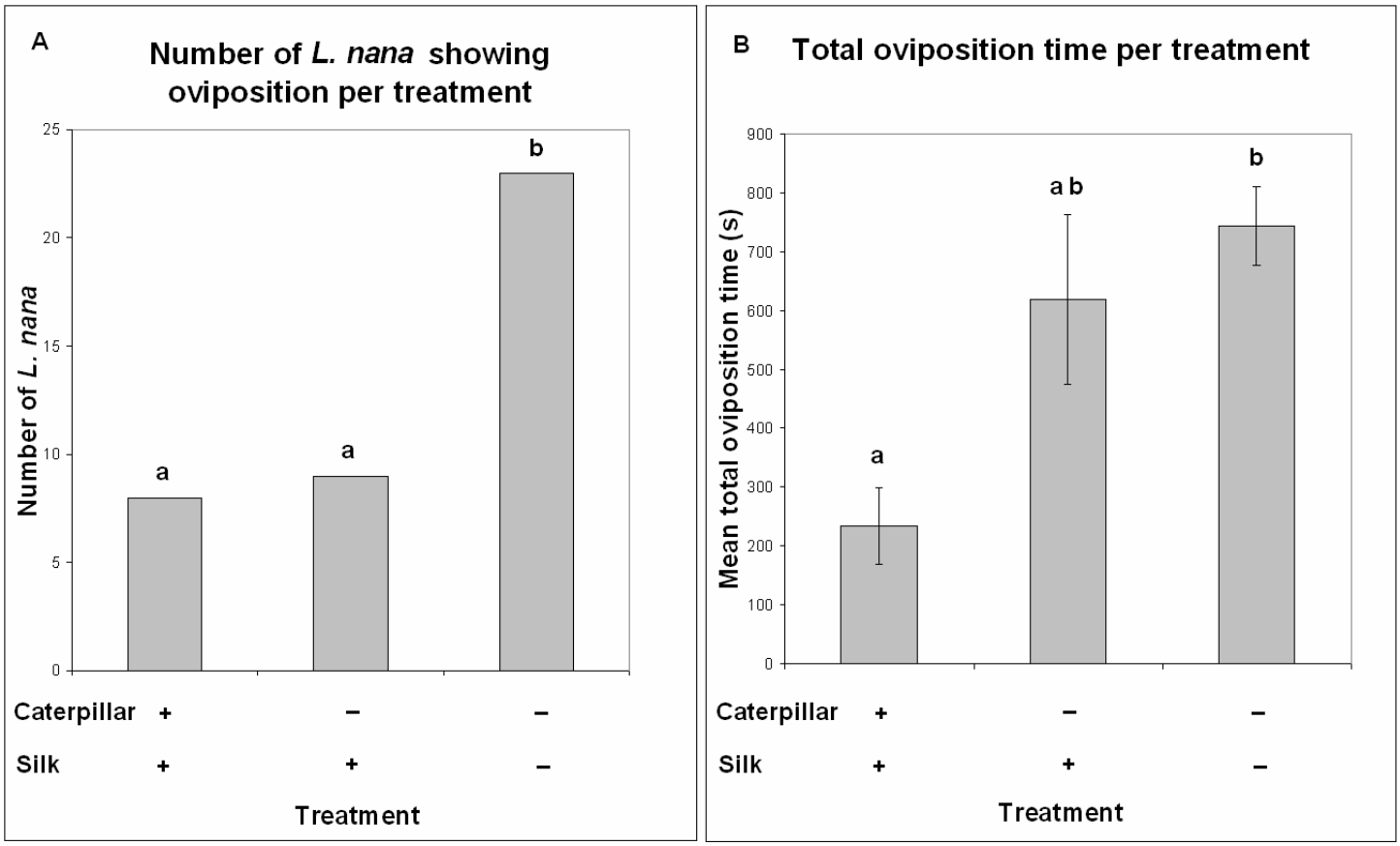

Figure 9: The number of replicates in which oviposition occurred (A) and the mean total oviposition time (B) per treatment. + represents present and – represents absent. Bars with different letters differ significantly ($p < 0.05$; Tukey (A) or Scheffe (B)). For A, the sample size per treatment = 25. For B, the sample size is the number of *L. nana* in A (8 for the first treatment, 9 for the second treatment and 23 for the third treatment). Means are shown (for B +/- SE).

Summarised, a much higher number of *L. nana* was able to oviposit the unattended, bare cocoons than both treatments of covered cocoons. Furthermore, the mean number of ovipositions and the mean total oviposition time were highest on the unattended, bare cocoons.

Mean time per oviposition

There was no difference in the mean time per oviposition between the different treatments ($F_{2,44} = 1.110$; $P = 0.339$), although the mean time per oviposition was lowest on the attended, covered cocoons (191 ± 41 seconds, compared to 422 ± 152 and 315 ± 39 seconds for the unattended, covered cocoons and unattended, bare cocoons respectively).

Cocoon abandoning measurements

Did *L. nana* go far away from the cocoons in the first 30 minutes after arrestment?

There was no difference in the number of *L. nana* per treatment that went far away (more then 4 cm) from the cocoons in the first 30 minutes after arrestment (Chi-square = 3.602; df = 2; $P = 0.165$). In 8 of the 25 replicates in the attended, covered cocoons-treatment, *L. nana* went far away from the cocoons. In 10 out of the 25 replicates in the unattended, covered cocoons-

treatment, *L. nana* went far away from the cocoons, whereas this number was 4 in the unattended, uncovered treatment.

Of the replicates in which *L. nana* went far away from the cocoons, for how long did they stay away during the first 30 minutes after arrestment?

Of the replicates in which *L. nana* went far, the mean time spend far away from the cocoons in the first 30 minutes after arrestment did not differ between the treatments ($F_{2,19} = 0.088$; $P = 0.917$). The mean time spend far was 381 ± 146 seconds for the attended, covered cocoons; 324 ± 125 seconds for the unattended, covered cocoons and 236 ± 122 seconds for the unattended, bare cocoons.

How often did *L. nana* go in and out the circle that contained the cocoons in the first 30 minutes after arrestment?

There was also no difference in number of times that the secondary hyperparasitoid left the cocoons (equal to the number of times going out of the circle that contained the cocoon cluster) between the different treatments (Chi-square = 4.234; $P = 0.120$; fig. 10), although *L. nana* left the bare cocoons less than the covered cocoons (fig. 10).

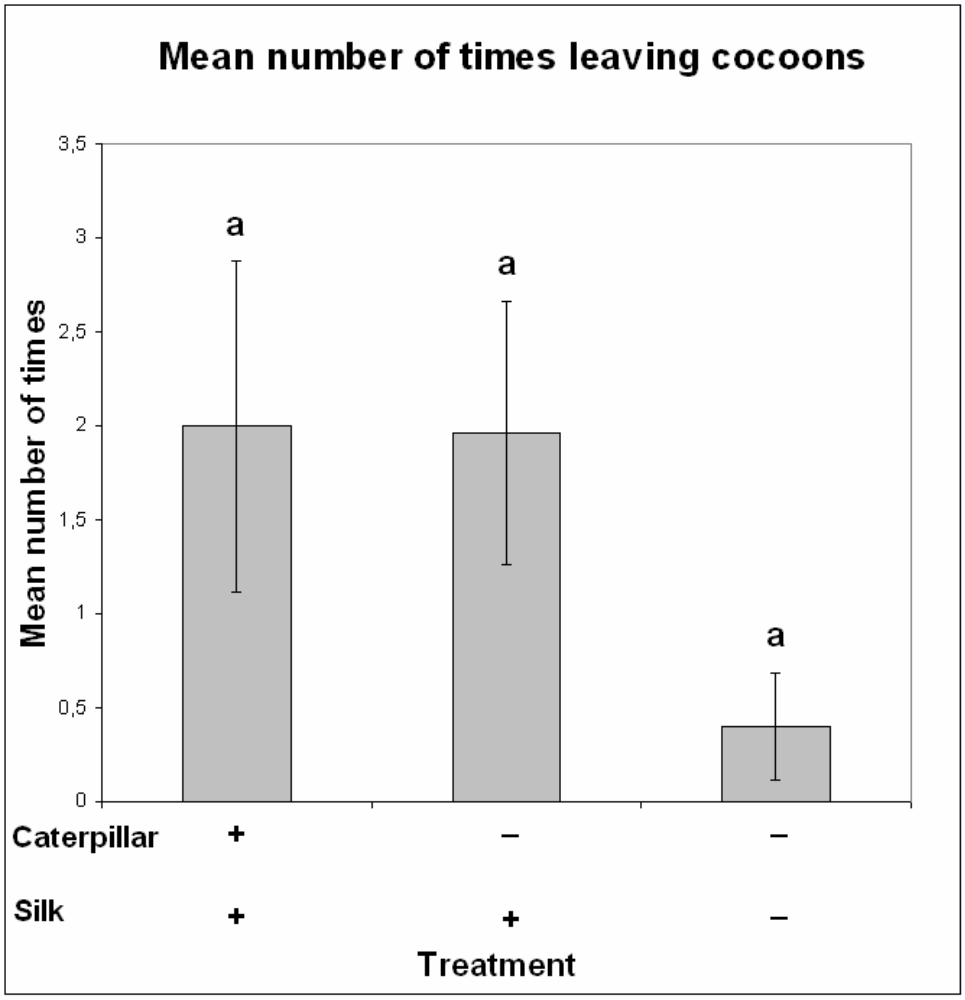

Figure 10: The mean number of times that *L. nana* left the cocoons (equal to the number of times that *L. nana* went out of the circle that contained the cocoon cluster). + represents present and – represents absent. Bars with different letters differ significantly ($p < 0.05$; Kruskal Wallis). Means are shown (+/- SE). Sample size is 25 per treatment.

These results of different tests aimed at analysing cocoon abandoning behaviour by the secondary hyperparasitoid (by walking away from these cocoons), indicated that there were no differences between the three treatments (attended and covered cocoons, unattended and bare cocoons, unattended and bare cocoons). Fewest *L. nana individuals* went far away from the cocoons in the treatment of the bare cocoons. Total time that *L. nana* spend far away from the cocoons (of the ones that went far) and the number of times that *L. nana* left the cocoons were also lowest in this treatment (fig. 10).

Experiment 4: Caterpillar survival

The mean number of days a caterpillar lived after emergence of the parasitoid larvae was 6.95 ± 0.47 days. Since the parasitoids in the cocoons are only available for hyperparasitation the first 5 days after the spinning of the cocoons (see hypothesis 5; Harvey et al. 2006), almost all caterpillars lived long enough to be able to guard the parasitoid cocoons against hyperparasitation (fig. 11). However, the larger the brood size, the shorter the caterpillar lived ($F_{1,19} = 6.573$; $P < 0.05$).

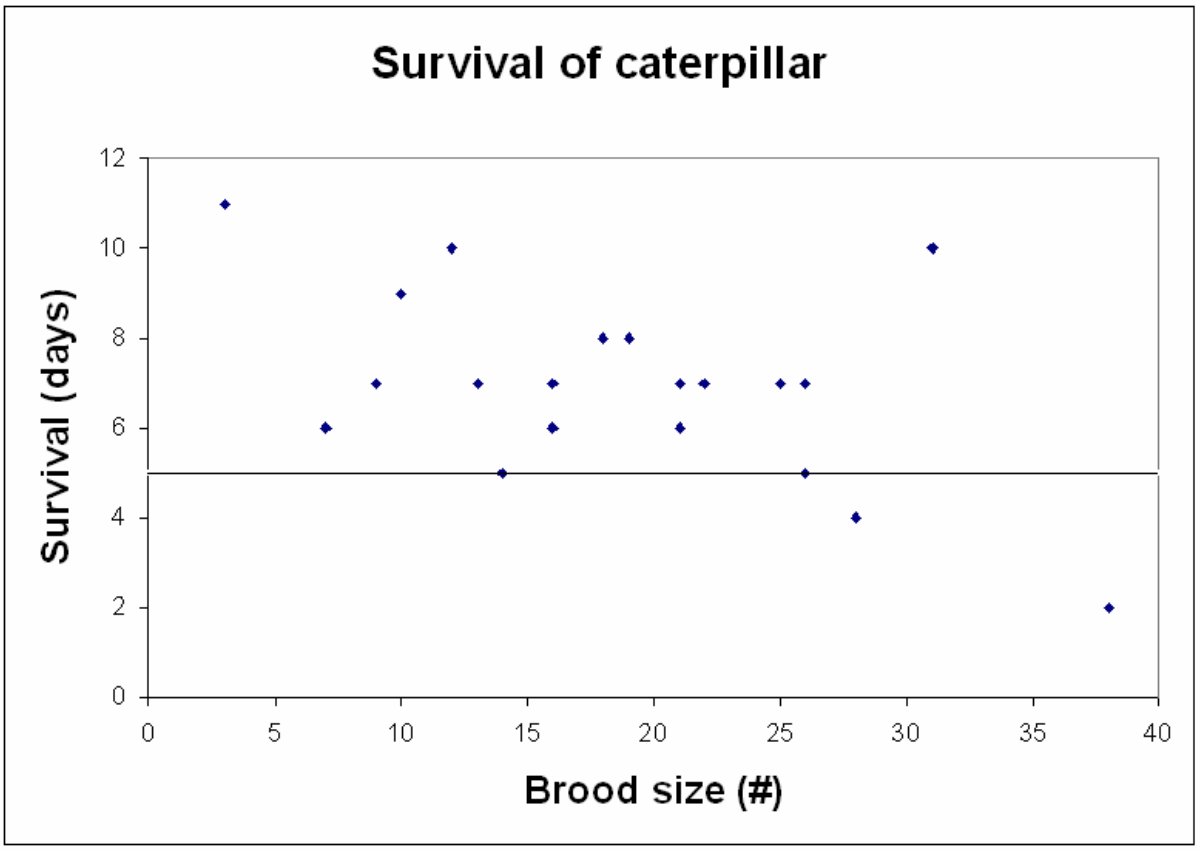

Figure 11: The survival (in days) of caterpillars after emergence of the parasitoids as a function of brood size (number of cocoons). The horizontal line at 5 days indicates the minimal lifespan necessary to be able to guard the vulnerable parasitoids.

Discussion

Parasitoids regulate their host during their development to increase parasitoid survival and fitness, and they influence the development, behaviour, physiology and morphology of the host (Vinson and Iwantsch 1980; Slansky 1986). The 'usurpation hypothesis' suggests that the primary parasitoid *C. glomerata* has the capacity to interfere with post-regression behaviour of the host and may have usurped some of the components of the host behaviour as a defence against predators and secondary hyperparasitoids (Brodeur and Vet 1994). For the 'usurpation hypothesis' to be accepted, the manipulated host must contribute to parasitoid survival during pupal development by protecting the pupae inside the cocoons from natural enemies (Brodeur and Vet 1994). In this study, this means that parasitized *P. brassicae* larvae must contribute to *C. glomerata* survival by protecting the parasitoid cocoons from hyperparasitism by *L. nana*. A few aspects seem to be important in the acceptance or rejection of the 'usurpation hypothesis' in this study system.

First of all, after emergence of the larval parasitoids from the host, the caterpillar must survive long enough to be able to protect the parasitoids from their natural enemies (e.g. predators, hyperparasitoids). As hypothesised, in this study it was shown that parasitized *P. brassicae* larvae easily survived long enough to be able to attend the parasitoid cocoons during the period (e.g. pupal development in cocoons) that they were susceptible to natural enemies. Of course, for the silk web to be protective, the caterpillar itself does not have to live for 5 days, only long enough to spin the silk web that covers the cocoons.

Secondly, it is important to realize that there could also be costs involved: the presence of the host can attract the secondary hyperparasitoids via the release of odours (e.g. kairomones). Parasitic wasps use chemical and physical cues from their host, or from the plants damaged by their host, to find suitable hosts (Vinson 1976; Vet and Dicke 1992). Although no studies have been able to determine what cues primary and secondary hyperparasitoids use to locate hosts, it is almost certain that olfactory cues associated with damaged plants and host-by products are involved (Buitenhuis 2004). For example, secondary hyperparasitoids can use the odours or visual cues of the host cocoons, but can also use odours and visual cues of the attached caterpillar or the additional silk web spun by the parasitized larvae. In contrast to this, in the experiments where *L. nana* was given a choice between two cocoon clusters, there was not a significant difference in the initial preference for an attended and/or covered cocoon cluster of *C. glomerata*. Furthermore, in the experiments where the foraging behaviour *of L. nana* was monitored, the time from entering the arena to behavioural 'arrestment' on a cocoon cluster by *L. nana* females also was not correlated with the presence or absence of a caterpillar and the additional silk layer overlying the cocoons.

Thirdly, and most importantly, for the usurpation hypothesis to be validated the presence of the parasitized caterpillar and/or its by-products must have a negative influence on hyperparasitism, and thus increase the chances of parasitoid survival. In this study two main effects associated with the parasitized host on hyperparasitism and parasitoid survival were analysed separately; these were the caterpillar (the presence, state or position of this caterpillar) and the presence of the silk web. Given that *L. nana* is a highly specialised secondary hyperparasitoid of fully cocooned parasitoid species in the genus *Cotesia* (Schwarz and Shaw 2000), with its main host perhaps being *C. glomerata* (Harvey and Witjes 2005), it was possible that neither of these aspects would influence parasitoid survival. This is due to the fact that *L. nana* is under rigorous selection pressure to find and parasitize hosts which are probably hard to find in nature. Therefore, abandoning a cocoon cluster could have a significantly negative effect on the fitness of a hyperparasitoid female if she is unable to locate cocoon clusters without attending caterpillars. In support of this argument, the presence of an attending caterpillar did not significantly lead to reduced hyperparasitoid emergence nor

did it have an influence on *C. glomerata* survival. By contrast, the presence of the additional silk web produced by parasitized caterpillars over cocoon clusters of *C. glomerata* was correlated with an increase in the number of *C. glomerata* offspring and a concomitant decrease in the number of *L. nana* offspring, irrespective as to whether a caterpillar was present or not.

The amount of time spent by female hyperparasitoids on (or very close to) cocoon clusters did not differ between the two treatments in all three choice experiments and there was also no significant difference in the number of *L. nana* offspring between the two treatments in all three choice experiments. It was hypothesised that *L. nana* would prefer to attack cocoons clusters that were most accessible (e.g. unattended and/or bare cocoon clusters), but this was not the case. Consequently, when given a choice, *L. nana* does not remain on unattended and/or bare cocoons for longer periods of time.

The behaviour of *L. nana* during a foraging bout was analysed separately. It was hypothesised that the behaviour of *L. nana* would be more disturbed when a caterpillar and/or silk web were present, which would result in being more prone to leave cocoon clusters under these conditions, combined with a shorter total oviposition time. In support of this hypothesis, the number of replicates in the observer experiment in which oviposition by *L. nana* occurred was significantly lower with both treatments of covered cocoons than with the bare cocoons, showing that the silk (at least partly) inhibits *L. nana* from parasitizing the cocoons. This result was supported by the lower number of ovipositions per replicate (of the replicates in which *L. nana* was able to oviposit) on the unattended covered cocoons compared to the bare cocoons, and the lower total oviposition time of *L. nana* (of the replicates in which *L. nana* was able to oviposit) on the attended covered cocoons compared to the bare cocoons. There was no significant difference in the mean time per oviposition between the three treatments in the observation experiment. In contrast to the hypothesis, there was not a significant difference in the tendency of wasps to leave cocoons in the differently treated cocoon clusters by *L. nana*. The behaviour of *L. nana* was also not found to be different between attended and unattended cocoons. Furthermore, once on the cocoons the behaviour of *L. nana* was little affected even when the caterpillar swung its headcapsule back and forth or physically contacted the hyperparasitoid, indicating that *L. nana* wasps are quite adapted to these behaviours from the host.

The result of a negative effect of the presence of a silk web on hyperparasitation of *C. glomerata* cocoons is opposite from the study by Tanaka and Ohsaki (2006), in which silk webs did not act as physical barriers against hyperparasitism (Tanaka and Ohsaki 2006). However, the differences in results with their study may be attributable to several important factors. First of all, Tanaka and Ohsaki used a different and much smaller hyperparasitoid species than was done here. Secondly, they only counted the number of hyperparasitoid offspring (an indirect measure of defence), whereas the number of *C. glomerata* was not recorded. Furthermore, although it was concluded that silk webs did not protect *C. glomerata* from hyperparasitism, it was suggested that silk webs do protect from hyperparasitation in larger cocoon clusters (Tanaka and Ohsaki 2006).

In summary, this study has shown that *C. glomerata* survival is higher when the parasitoid cocoons are reinforced with an extra layer of silk by the parasitized caterpillars, and that the number of offspring produced by *L. nana* is concomitantly reduced in this situation. Furthermore, oviposition behaviour in *L. nana* was negatively affected when the wasps experienced silk-covered cocoons. Consequently, *L. nana* is more efficient at parasitizing bare parasitoid cocoons than covered cocoons, and thus the silk web was an impediment in hyperparasitism by *L. nana*. However, in the choice experiment *L. nana* did not prefer the bare cocoons. So, although *L. nana* has a more difficult time parasitizing cocoons covered by

a silk web, when given a choice *L. nana* does not prefer the bare cocoons compared to the covered cocoons. How can we explain these results?

L. nana is a highly specialised secondary hyperparasitoid of fully cocooned parasitoid species in the genus *Cotesia* (Schwarz and Shaw 2000), with its main host perhaps being *C. glomerata* (Harvey and Witjes 2005). In nature, a female secondary hyperparasitoid does not find a lot of cocoon clusters in her life. When she finds one, she will be very determined in parasitizing this cocoon cluster, since the chances of her finding another cluster are not very high (J.A. Harvey, personal communication). On the other hand, *C. glomerata* does not want to be hyperparasitized, or eaten by a predator. So *C. glomerata* has evolved ways to protect itself from natural enemies, of which the usurpation of the caterpillar's behaviour is a very important one (Brodeur and Vet 1994). If the presence of the attending caterpillar, or the covering silk web, would lead to abandoning of the cocoon cluster by *L. nana*, this could have a significantly negative effect on the fitness of the hyperparasitoid female. To prevent this, female *L. nana* should be very persistent in trying to parasitize *C. glomerata* cocoons, whether attended by a caterpillar and covered by a silk web or not. In support of this, in this study it was shown that *L. nana* did not have a tendency to leave cocoons that were attended by a caterpillar or covered by a silk web, or prefer unattended or bare cocoons in a choice set-up.

However, the time necessary to parasitize host cocoons is also very important. The presence of the caterpillar or the silk web can make parasitation of host cocoons more difficult for *L. nana*. The longer the parasitation takes, the more likely it is that the female will be disturbed, for example by predators, weather conditions like rain or other animals that in some way disturb the female hyperparasitoid. In this study it was shown that within a timeframe of 6 hours, the presence of the silk web decreased hyperparasitation. How could the silk web covering the parasitoid cocoons have such an influence? Most importantly, the silk provides a physical barrier between the parasitoid cocoons and the hyperparasitoid. The silk web can prevent hyperparasitoids from detecting the quality of the host, because hyperparasitoids normally assess the developmental stage or physiological state of the host by direct antennal contact (Brodeur and Vet 1994; Tanaka and Ohsaki 2006). Furthermore, the hyperparasitoids' ovipositor must touch the cocoons to be able to lay an egg on the cocoon's surface, and cocoons with a space between them and the silk web might therefore avoid hyperparasitation (Tanaka and Ohsaki 2006). *L. nana* could chew a hole in the silk layer to crawl through, but this chewing will take time. Time in which the female hyperparasitoid can be predated on, or time in which she can be disturbed from oviposition by weather conditions such as rain or other animals.

The results of this study provide partial support for the 'usurpation hypothesis' in this multitrophic association. The presence of the silk web was correlated with an increase in parasitoid survival. However, opposite to the major part of the 'usurpation hypothesis', the attending caterpillar did not increase parasitoid survival. Furthermore, not all caterpillars spin a silk web that covers the cocoons. In a study by Tanaka and Ohsaki (2006), about 25% of the tested caterpillars did not spin a silk web at all. About 25% spun a totally covering silk web (100% cover), but the remaining 50% made a silk layer varying from only 10% cover to 90% cover (Tanaka and Ohsaki 2006). Therefore, the survival and presence of the host after parasitoid emergence did not necessarily lead to a higher parasitoid survival. The 'usurpation hypothesis' is therefore only partially accepted on the basis of these results.

Although the 'usurpation hypothesis' can be partially accepted, it is probable that the usurpation of host behaviour is much more aimed at protection from generalist predators than from more specialized hyperparasitoids. Many predators are capable of feeding on a variety of prey species (Godfray 1994; Harvey and Witjes 2005), and if they are repelled by the attending caterpillar or the silk web that was spun by the caterpillar to cover the cocoons, they can easily look for alternative prey (other types of insects). The expected influence of the

attending host on parasitoid survival when predators are involved is confirmed by the study by Kester and Jackson (1996), in which the predatory bug, *Jalysus wikhami*, attacked fewer *C. congregata* pre-pupae and pupae in cocoons that were attached to *Manduca sexta* larvae than detached siblings. Moreover, host-finding and reproduction are directly linked in parasitoids, whereas in predators they usually are not. Furthermore, many parasitoids, including *L. nana*, have limited host ranges (Godfray 1994; Harvey and Witjes 2005). *L. nana* only attacks wasps in the braconid subfamily Microgastrinae, in particular *Cotesia* species which pupate in an exposed location on the host foodplant (Schwarz and Shaw 2000). Therefore, it is likely that they have experienced long periods of co-evolution with these parasitoids and their secondary hosts. If they would abandon their host when a caterpillar would attend them, this would be a very costly decision, decreasing their chance for reproduction. *L. nana* is therefore expected not to be very prone to abandon potential hosts, which was confirmed by the results of this study.

Outlook for future research

To gain more information about the 'usurpation hypothesis' in this study system, more different treatments could be tested against each other in the choice and behavioural observation experiments, e.g. the addition of an attended, bare cocoon cluster. Most importantly, the time-limiting effect on hyperparasitation should be tested. Maybe the effect of the caterpillar and the silk web are much more obvious when *L. nana* has a shorter time to parasitize the cocoons, whereas the effects are almost gone when *L. nana* has an unlimited parasitation time. Hyperparasitation by *L. nana* could be compared between treatments in which *L. nana* has different amounts of time to parasitize the cocoons. This could give more insight in time-limiting effects on hyperparasitation within different treatments. These time-limiting effects on hyperparasitation should most importantly be tested in the field, since secondary hyperparasitoids can be easily disturbed from oviposition there, as a result of predators or other animals, and weather conditions such as rain. It would also be very interesting to test the 'usurpation hypothesis' with an insect predator that feeds on *C. glomerata* cocoons. In this way, it can become clear at which natural enemies, predators or hyperparasitoids, the usurpation of host behaviour by *C. glomerata* is mainly aimed. Finally, the hypothesis could be tested on a wider variety of associations involving parasitized hosts, microgastrine braconids and their secondary hyperparasitoids. Some hosts, such as *Manduca sexta*, remain far more active after parasitoid egression than do *P. brassicae* larvae. Furthermore, some microgastrines, such as *C. congregata*, attach cocoons to the dorsal side of the host but remain physically detached from one another. Further studies will reveal the utility of the usurpation hypothesis against hyperparasitoids in these kinds of associations.

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