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Brochosome influence on parasitisation efficiency of *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae) egg masses by *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae)

HANS-PAUL VELEMA[1,2], LIA HEMERIK[2], MARK S. HODDLE[1] and ROBERT F. LUCK[1] [1]Department of Entomology, University of California, Riverside, U.S.A., [2]Biometris, Department of Mathematical and Statistical Methods, Wageningen University, the Netherlands

Abstract. 1. Many cicadellid females in the tribe Proconiini (Hemiptera: Cicadellidae) cover their egg masses with specialised, usually rod-shaped, brochosomes as the eggs are being laid. The brochosomes are produced in Golgi complexes in the Malpighian tubules of Cicadellidae. In contrast to the gravid females, adult males, pre-reproductive adult females, and nymphal males and females produce specialised, usually spherically shaped brochosomes. Brochosomes are also used to cover the external surfaces of nymphs and newly moulted adult males and females.

2. The function of the brochosome covering the egg masses is unknown but various hypotheses have been suggested, including protecting the eggs against pathogens, predators, and parasitoids. Based on preliminary observations of *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae) parasitising the eggs of the cicadellid, *Homalodisca coagulata* (Say), it is speculated here that brochosomes covering an egg mass hinder parasitisation of eggs by *G. ashmeadi*. This hypothesis was tested by observing *G. ashmeadi* females foraging on leaves with *H. coagulata* egg masses heavily covered with rod-shaped brochosomes vs. those lacking brochosomes.

3. Cox's proportional hazards model was used to evaluate the probability, per unit time, that a female *G. ashmeadi* displayed the sequence of behaviours that ended in successful oviposition as influenced by five variables: (a) presence or absence of brochosomes on an egg mass, (b) the leaf surface, upper or lower, being searched by the parasitoid (the egg masses are laid in the parenchyma on the lower leaf surface), (c) the parasitoid's previous ovipositional experience, (d) egg mass size, and (e) the parasitoid's age.

4. Brochosomes significantly decreased oviposition efficacy of *G. ashmeadi* females. Scanning electron microscopy showed that females exposed to brochosome-covered egg masses had brochosomes adhering to their tarsi, legs, antennae, and eyes, all of which prompted extensive bouts of grooming.

Key words. Behaviour, brochosomes, egg parasitoid, glassy-winged sharpshooter, grooming, proportional hazards model, survival analysis, time budget.

Introduction

Many cicadellid females in the tribe Proconiini (Hemiptera: Cicadellidae) cover their egg masses and surrounding leaf

Correspondence: Mark S. Hoddle, Department of Entomology, University of California, Riverside, CA 92521, U.S.A. E-mail: mark.hoddle@ucr.edu

area with specialised, usually rod-shaped, brochosomes during oviposition (Hix, 2001; Rakitov, 2004). This particulate material is produced in the Golgi complexes of a gravid female's Malpighian tubules. It is exuded in a liquid drop from the female's anus prior to oviposition and the female moves the drop from her anus to each of her forewings with her metathoracic legs where the droplets dry to become a white, chalky patch (Hix, 2001; Rakitov, 2002a, b, 2004). In *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae), the glassy-winged sharpshooter, brochosomes are moved from forewing patches to the oviposition scar and surrounding area on the underside of a leaf after each pair of eggs are laid, or on a solitary egg which can only be the terminal egg (Hix, 2001). The eggs are inserted through a slit in the epidermis of the lower leaf surface into the parenchyma tissue, which is then followed by the female spreading brochosomes over the oviposition scar and surrounding area. This process continues with each egg-laying bout resulting in an egg mass of from one to 30 elongated eggs, usually laid adjacent and parallel to one another (Hix, 2001; Al-Wahaibi, 2004). Several hours after the egg mass is laid, the eggs swell to form a blister on the underside of the leaf surface (Al-Wahaibi, 2004). It is this blister and an area extending several millimetres beyond its margin that is covered with brochosomes (for brochosome-free and brochosome-covered egg masses see Fig. 1).

The rod-shaped brochosomes covering an egg mass differ from those produced by a newly moulted nymphal male or female, or by a newly moulted adult male or non-gravid female. A newly moulted sharpshooter also exudes the spherical brochosomes in droplets from its anus (Rakitov, 2002a, 2002b, 2004), which are then spread over the insect's integument with its metathoracic legs after each moult (Rakitov, 2002b). Brochosomes may prevent the insect from becoming entrapped in its own aqueous excrement (Rakitov, 2002b). The same cells that produce the spherical brochosomes in the nymphal and newly moulted pre-gravid female may switch to producing rod-shaped brochosomes in gravid female Proconiini (Rakitov, 2002a, 2004). The adaptive significance of rod-shaped brochosome coatings is unknown (Rakitov, 2002a). It is speculated that they may protect eggs against pathogens, predators, and parasitoids (Rakitov, 2004). None of these natural enemy protection hypotheses have been tested (Hix, 2001; Rakitov, 2004).

While conducting behavioural observations with *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae), an egg parasitoid of *H. coagulata* and several other Proconiini sharpshooters (Huber, 1988), a whitish residue on the frons, antennae, and legs of some of the searching females was noted. The presence of this whitish residue was associated with the presence of rod shaped brochosomes on egg masses that had been antennated by female parasitoids. Parasitoids with this residue also spent substantial time preening their antennae and legs to which this residue adhered. Its presence appeared to interfere with parasitoid searching behaviour. This had been observed in other species (Jones, 2002). In nature, *H. coagulata* egg masses vary in the amount of brochosomes that cover them, ranging from egg masses with little or no brochosomes to those that are heavily covered with them (M. S. Hoddle, R. F. Luck and D. J. W. Morgan, pers. obs.). Given these initial observations, it was decided to conduct a set of behavioural experiments to test whether egg masses heavily covered with brochosomes hindered successful egg parasitisation by *G. ashmeadi*. Thus a set of observations were initiated to test (i) whether the presence of brochosomes on an egg mass decreased the efficacy of the parasitoid, (ii) whether the parasitoid was more likely to search the upper leaf surface when the egg mass was heavily covered with brochosomes (*H. coagulata* egg masses are laid in the parenchyma on the underside of the leaf), (iii) whether the parasitoid's previous ovipositional experience increased its efficacy in exploiting an egg mass, (iv) whether the parasitoid's efficacy was decreased with the size of the egg mass, and (v) whether parasitoid age affected its parasitisation efficacy.

Materials and methods

Homalodisca coagulata *cultures*

Experimental egg masses were obtained from an *H. coagulata* culture, initiated with adults collected from citrus trees (*Citrus* spp.) located at Agricultural Operations, Citrus Experiment Station, University of California, Riverside California, U.S.A. The adults were maintained on euonymus plants (*Euonymus japonica* L.; Celastraceae)

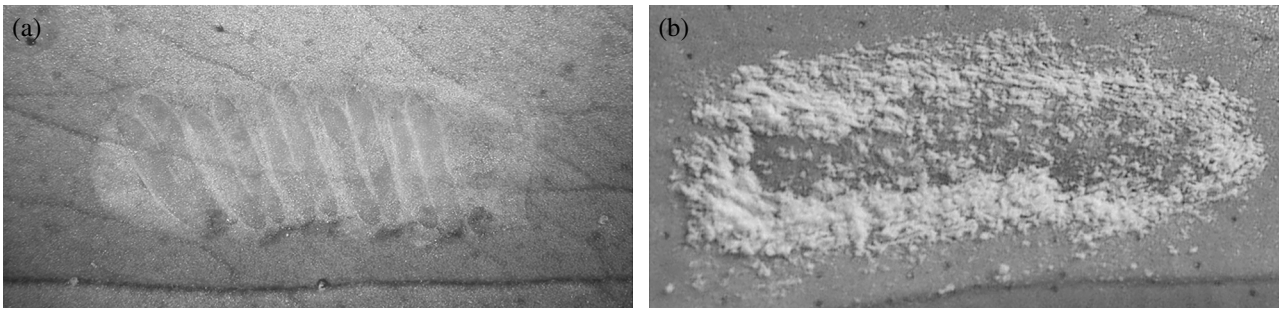

Fig. 1. An egg mass of *Homalodisca coagulata* without brochosomes (a) and with brochosomes (b).

in cages (30 × 61 × 60 cm) constructed from aluminium framing. One-millimetre mesh aluminium screening covered the sides and top of the cage and a clear Plexiglas piece (3 mm thick) comprised its front panel. Adult *H. coagulata* and host plants were added, or leaves with *H. coagulata* egg masses were extracted through a sleeved 30 × 30 cm opening in the Plexiglas front panel. Additional adult *H. coagulata* were field collected as needed and added to the culture periodically when adult numbers decreased below about 50 individuals. The *H. coagulata* colony was maintained in a temperature cabinet at 28 °C and LD 16:8 h photophase.

The euonymus plants were checked daily for newly laid egg masses. Each leaf with an egg mass was picked from the plant and the leaf petiole was inserted into a 1.5-cm long slit cut in a 30 × 20 × 0.7 cm piece of foam rubber enclosed in a 30 × 20 × 8 cm plastic container filled 1 cm deep with water. A lid closed the container to prevent *Gonatocerus* spp. from gaining access to the harvested egg masses. The containers were held in a temperature controlled room (25.5 ± 1.1 °C). For the experiments, egg masses less than 72 h old were used as *G. ashmeadi* prefers this egg age for oviposition (Irvin & Hoddle, 2005). Occasionally fungal growth began in the containers. It was controlled by washing the containers and foam rubber pieces with 75% ethanol followed by a thorough water rinse.

Gonatocerus ashmeadi *cultures*

Parasitoids were obtained from parasitised *H. coagulata* egg masses on lemon or grape (*Vitis* spp. Vitaceae) leaves collected from plants located at Agricultural Operations, University of California, Riverside, California, U.S.A. Field-collected material was occasionally augmented with *G. ashmeadi* reared by the California Department of Food and Agriculture, Mt Rubidoux Facility, Riverside, California, U.S.A. These parasitoids were mass reared on *H. coagulata* eggs laid in *Chrysanthemum* sp. (Asteraceae) or *E. japonica* leaves. Petioles of excised leaves with parasitised egg masses were inserted into slits cut into a piece of water-saturated foam rubber and maintained as the *H. coagulata* egg masses above, in a separate container. The parasitised egg masses were checked daily for dark-coloured parasitoid pupae within an egg mass which appeared approximately 8–10 days after parasitisation. Leaves bearing parasitised egg masses were removed from the containers and placed on a moist filter paper disk lining the bottom of a sealed Petri dish and the parasitoids were allowed to emerge. All emerged parasitoids used in experiments were 24–72 h old, fed honey upon emergence, and exposed to unparasitised *H. coagulata* egg masses in the experiment.

Behavioural experiment

The experiments in this study compared two classes of brochosome coverings on *H. coagulata* egg masses – those free or nearly free of brochosomes vs. those heavily covered with brochosomes. To compare the effects of brochosome-covered vs. brochosome-free egg masses on the searching and oviposition behaviours of a female *G. ashmeadi*, an *E. japonica* leaf was attached, lower leaf surface up, to the inside bottom of a glass Petri dish using Duco Stik-Tak® (*H. coagulata* egg masses are laid on the underside of leaves). The Petri dish bottom was held at a 45 ° angle to facilitate viewing a parasitoid's searching and parasitisation behaviours. All experimental egg masses were uniquely labelled. A parasitoid's foraging behaviour on a leaf surface or egg mass was viewed and recorded using a video camera (MTV-7366 ScienceScope, Chino, California) mounted to a dissecting microscope (Leica MZ12, Wetzler, Germany). The image was relayed to a video monitor and recorded on videotape for subsequent analysis. The time, in seconds, that each parasitoid spent in one of six behaviours was recorded: (1) walking – parasitoid walking and antennating leaf surface; (2) antennating the egg mass – the parasitoid rapidly drums eggs with the tips of its antennae; (3) drilling – the parasitoid pierces an individual egg within an egg mass with its ovipositor while thrusting its abdomen up and down in a probing motion; (4) oviposition – characterised by a 'bouncing' movement of the parasitoid's abdomen indicating that an egg had been deposited inside the host egg; (5) grooming – the parasitoid cleans itself by rubbing, scraping, or nibbling one or more of its body parts while stationary (Basibuyuk & Quicke, 1999); and (6) resting – the parasitoid stands motionless. In a few instances (12% of the individuals exposed to brochosomes) a parasitoid ceased searching and laid down on a leaf that was heavily covered with brochosomes, for at most 6 s. This behaviour was classified as resting.

An observational bout was defined as the period of time beginning with the parasitoid initiating its search of a leaf and ending when it left a leaf or when we terminated our observation. Each parasitoid was used only once for each observational trial. A summary of behavioural information was obtained from the videotapes by entering data into an Excel spreadsheet using a custom-written Visual Basic program. For each of the six behavioural events, its start time in seconds was denoted, and each behavioural record was saved separately. For example, a parasitoid repeated some behaviours, e.g. grooming, several times during an observational bout and the start of each of these events was denoted as a separate record. The behaviours of 56 female *G. ashmeadi* – 45 of which were exposed to brochosome-covered egg masses and 11 were exposed to brochosome-free egg masses – were analysed. The total number of behavioural transitions was approximately the same in both samples (see Fig. 4).

Interruption experiment

To determine whether abdominal 'bouncing' movements by a female *G. ashmeadi* are associated with oviposition, nine trials were conducted in which an ovipositing female

was interrupted during egg laying. An *H. coagulata* egg mass containing at least three eggs was exposed to a single female *G. ashmeadi* ($\approx$ 24 h old) and the parasitoid's behaviour was observed and recorded. The female was allowed to parasitise one to three eggs before it was interrupted at the onset of abdominal 'bouncing' by dislodging it from the egg with a pencil tip. Eggs in which 'bouncing' was observed or 'bouncing' had been interrupted were noted by marking the egg mass with an indelible marker. The leaves with exposed egg masses were kept in a separate container with water and foam rubber as described in the section on *H. coagulata* cultures. The egg masses were checked daily for parasitoid development. Prior to parasitoid emergence, leaves bearing the egg masses were removed from the piece of foam rubber, and placed on moist filter paper disks lining the bottom of a closed Petri dish for parasitoid or *H. coagulata* emergence. The size, shape, and location of the exit holes were used to indicate whether a parasitoid or a *H. coagulata* nymph had emerged. Eggs that failed to hatch were dissected and checked for dead parasitoids or *H. coagulata* nymphs.

Scanning electron microscopy

During preliminary behavioural observations, a white material that had adhered to parasitoids foraging on leaves with brochosome-covered egg masses was noted. Scanning electron microscopy was used to determine if this material was made up of brochosomes. To prepare a *G. ashmeadi* female for electron microscopy, parasitoids were allowed to encounter egg masses coated with brochosomes and, after accumulating a sufficient amount, parasitoids were collected into vials (2 by 8 cm), which were sealed with cotton wool and placed immediately in liquid nitrogen to kill the parasitoids. Individual parasitoids were prepared for scanning electron microscopy in one of two ways: parasitoids were coated with gold–palladium either (1) directly within 24 h of death, or (2) after first being freeze dried. The whitish material on parasitoids was compared: (1) to that covering an *H. coagulata* egg mass and (2) to the brochosome patch on the forewings of female *H. coagulata*. Unfixed leaves with brochosome-coated egg masses were either fresh or desiccator-dried before being subjected to scanning electron microscopy. Unfixed *H. coagulata* tegminae with a white brochosome patch were also examined with scanning electron microscopy. Selected examples of these specimens were photographed using a Philips XL 30 scanning electron microscope at the University of California, Riverside.

Behavioural transitions

The behavioural records of parasitoids foraging on brochosome-free or brochosome-covered egg masses were summarised from the video tapes separately as transition matrices (see Fig. 4). To assess whether the presence of brochosomes significantly affected the behavioural transitions, the statistical test proposed by Haccou and Meelis (1992) was performed. To gain insight into the organisation of the behaviour, behavioural kinetograms were also constructed (see Fig. 5). This allowed the visualisation of differences in the behavioural sequences between the parasitoids exposed to brochosome-free or brochosome-covered egg masses. For analysis, it was assumed that behavioural sequences manifested by the individual parasitoids did not differ significantly within a given treatment (e.g. brochosome-covered egg masses) and the data were pooled for all individuals for that treatment. In each transition matrix, the values on the diagonal are logical zeros, as a behaviour cannot logically follow itself in such a record. Goodman's (1968) iterative proportional fitting method was used to find the expected values for the non-zero matrix elements and used a log-likelihood ratio test (G-test) to evaluate their statistical significance for the overall matrix table. Throughout the procedure, Yates's correction was applied for continuity. If the deviations in the overall matrix table were significant, then the significant transitions were found by collapsing the full matrix table into a 2×2 matrix around each transition. The significance of these individual tests was adjusted to a table-wide level of 5% with the sequential Bonferroni method (Rice, 1989). The resulting kinetograms were constructed by including only those behaviours with a frequency of occurrence that positively deviated from the expected value (i.e. random transitions, see also Field & Keller, 1993; Wang & Keller, 2002). The goal of this analysis is to provide a clear picture of how the observed suite of behaviours differs when a parasitoid forages on a brochosome-free or brochosome-covered egg mass.

Survival analysis

Survival analysis (Kleinbaum, 1996) was used to determine whether the initiation or cessation of an event, i.e. its failure time, was affected by one or more of the five covariates (see below). With survival analyses, the time until an event of interest occurs is termed its failure time. This analysis was originally used in medical research and the event of interest was usually associated with a death (Kleinbaum, 1996), hence the term failure time. In this study, the event of interest was considered to be the start or end of a particular behaviour by a parasitoid. These events define interval times and behaviour times, as shown in Fig. 2.

The time from the start of an observation until the start of a particular behaviour is called its interval time. Once this event has occurred, a new interval time begins the moment this behaviour ends, i.e. a renewal point occurs. The behaviour's duration is the time from its initiation until the behaviour ceases, which, by convention, is referred to as the behaviour time (e.g. walking behaviour, Fig. 2). Thus, each start of a particular behaviour is a renewal point for this behaviour time. The tendency to start or stop a particular behaviour is referred to as its hazard rate, and can be

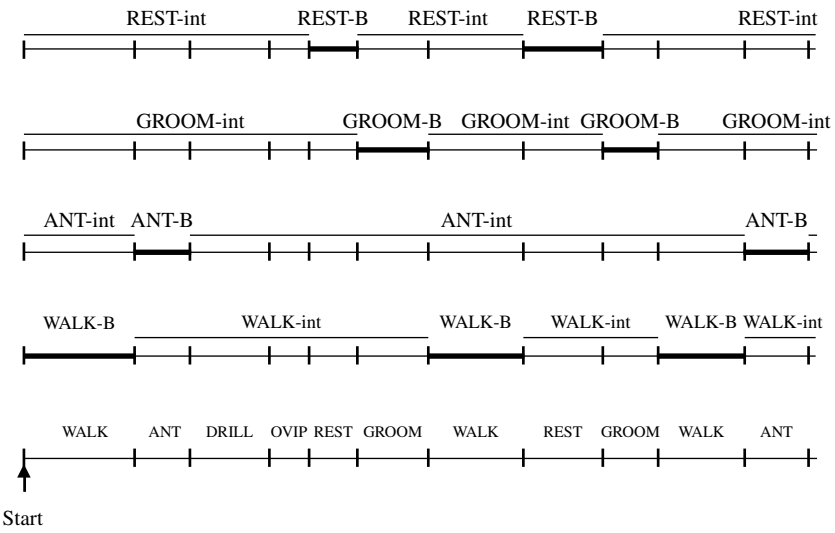

Fig. 2. Schematic representation of interval time (int) and behaviour time (B) for four behaviours in the behavioural experiment. The lower scheme shows a part of a fictitious behavioural record (ANT, REST, OVIP denote antennate, rest, and oviposit respectively).

estimated from the data using survival analysis (Kleinbaum, 1996). The hazard rate is the probability per unit time that an event of interest occurs, conditionally on it not having occurred yet. In some cases, an observation period ends before the event of interest has occurred, in which case the time is recorded as a censored failure time. For such censored events, the actual failure time lasts at least as long as the censored time. Thus, the censored time gives a minimum estimate of the failure time for that behaviour (Bressers *et al*., 1991). Censored events arose because an experiment was terminated after a set period of time. By contrast, when a parasitoid abandoned an experimental leaf; the time for the behaviour that had been initiated was denoted as its failure time.

Covariates

Factors such as parasitoid age, egg mass age, or host plant could not be standardised consistently across every trial. Such non-standardised factors are referred to as covariates (Haccou & Hemerik, 1985). Survival analysis allows inclusion of their effect on hazard rates by plotting Kaplan–Meier survival curves for different values of each covariate (Kalbfleisch & Prentice, 1980; Kleinbaum, 1996). The Kaplan–Meier estimate is a product of survival probabilities:

$$\mathrm{SKM}(t) = \prod_{t_i<t} \frac{r(t_i) - d(t_i)}{r(t_i)}, \qquad (1)$$

where r is the number of parasitoids at risk (i.e. still waiting for an event to happen just prior to t_i) and d is the total number of events that occurred at time t_i (the resulting survival curve is a step function that ranges from 1 to 0 with a decrease at each event). In a preliminary exploratory data analysis, the plots were used that gave the estimated hazard rates (i.e. the log-survival) for different values of fixed covariates to determine whether these observed hazard rates differed from one another (Kalbfleisch & Prentice, 1980; Kleinbaum, 1996).

Those covariates that showed clearly different Kaplan–Meier hazard rates were included in our proportional hazards analysis. Only those behavioural records that contained values for all five of the covariates were analysed. Their inclusion in the survival analysis was based on their apparent influence on the hazard rate of at least one behavioural category. Only with Cox's proportional hazards model can the magnitude of an effect be estimated (see below).

The five covariates that were included were: (1) The presence (coded 1) or absence (coded 0) of brochosomes on an *H. coagulata* egg mass (BROCH). (2) The number of ovipositions prior to the event under consideration (OVIP): coded as 0, 1, or 2^+ for, respectively, zero, one, or two or more previous ovipositions on the current leaf. Too few replicates were available in each of the ovipositional categories of two or more ovipositions to code them individually; therefore they were lumped together and treated as a single category. (3) The size of an egg mass (EMS) was either small (4–10 eggs) or large (11–16 eggs), coded 0 and 1 respectively. Too few replicates of each possible egg mass size were available to code each egg mass size separately. (4) The parasitoid's age (AGE): coded 0 if the wasp was $\leq$ 24 h old and 1 if it was $>$ 24 h old. All wasps were less than 72 h old. (5) The leaf side on which the behaviour occurred (SIDE): coded 0 if the parasitoid searched on the side of the leaf with the egg mass (egg masses are laid on the underside of leaves) or 1 for the side of the leaf without the egg mass. Switching leaf sides occurs while the parasitoid is walking; therefore, this covariate is time dependent and can change between 0 and 1 while the parasitoid is walking. During all other behaviours the value for this covariate is constant (i.e. either 0 or 1).

Cox's proportional hazards model

Cox's proportional hazards model (Cox, 1972) was used to test whether the presence or absence of brochosomes on an egg mass influenced the probability of beginning or

ending of a behavioural event, given the value of the other four covariates. The hazard function has the following form:

$$h(t, X(t)) = h_0(t) \exp\left[\sum_{i=1}^{p} \beta_i X_i(t)\right], \quad (2)$$

where $h(t, X(t))$ denotes the hazard rate at time t, given the influence of a collection (vector) of specified covariates, $X(t)$, that are either all fixed, all time dependent, or a mixture of the two. The baseline hazard function, denoted as $h_0(t)$, is multiplied by an exponential function that expresses the multiplicative effect of the $X_i(t)$ covariate, where $i = 1, \ldots, p$, multiplied by its corresponding regression parameter β_i. If a particular covariate, X_i, does not influence the hazard rate, then β_i does not differ significantly from 0. Under such circumstances, the baseline hazard is unaffected [$\exp(0) = 1$].

To analyse the data, the regression coefficients ($\hat{\beta}_i$) and the baseline hazard were estimated simultaneously by maximising the partial likelihood (Kalbfleisch & Prentice, 1980), using SPSS (version 10.1) and an APL program (Haccou & Hemerik, 1985). These estimates for the regression coefficients were then used to compute a hazard ratio (HR), which describes the effect of the covariate. If only one binary exposure variable is considered, coded 0 or 1, with estimated regression coefficient $\hat{\beta}$, the effect simplifies to

$$\mathrm{HR} = \frac{h(t,1)}{h(t,0)} = \frac{h_0(t)e^{\hat{\beta}}}{h_0(t)} = e^{\hat{\beta}}. \quad (3)$$

Hence, the hazard rates at different covariate values differ only by this multiplication factor $\exp\hat{\beta}_i$ (proportionality assumption).

The Z statistic is used to test whether $\hat{\beta}_i$ differs significantly from 0 and is calculated as $(\hat{\beta}_i/\mathrm{SE})^2$. If the number of observations approaches infinity, the Z statistic approximates a χ^2 distribution with p degrees of freedom (Haccou & Hemerik, 1985). The Cox's proportional hazards model can be visualised by drawing cumulative hazard plots, the slopes of which show how the hazard rate changes with time.

Residual plots

To test the overall fit of Cox's proportional hazards model, the negative log survival of the residuals were plotted against the values for the residuals. If the proportionality assumption holds, these plots give a 45 ° line through the origin for all covariate values. Note that this visual test can only be performed for fixed covariates (Kalbfleisch & Prentice, 1980).

Results

Scanning electron microscopy

Photographs clearly indicate that *H. coagulata* brochosomes rubbed from wing spots to cover the egg masses readily adhere to various body parts of a foraging parasitoid as shown in Fig. 3. Brochosomes can be seen on a *H. coagulata* forewing (Fig. 3a), attached to the tip of the parasitoid's antennae (Fig. 3b), and on the eye (Fig. 3c) and leg (Fig. 3d). Despite the careful handling of parasitoids exposed to a brochosome-covered egg mass, some of the brochosomes were dislodged while killing and preparing parasitoids for scanning electron microscopy. Thus, the scanning electron micrographs showed fewer brochosomes attached to a parasitoid's body parts than was observed during video recording.

Parasitoid searching behaviour

When a female *G. ashmeadi* encountered a leaf, she began antennating its surface by drumming it with her antennal clubs as she walked on the leaf (= WALK). During antennation (= ANT), both antennae moved alternating in vertical, anterio-posterior arcs relative to the long axis of the parasitoid's body when she was drumming the leaf surface.

When a female encountered an egg mass, manifested as a leaf blister with *H. coagulata* eggs beneath (Fig. 1a,b), she walked across its surface while drumming it as she moved across the leaf blister (= ANT behaviour, Figs 4 and 5) while reducing her forward speed and increasing her attennation rate. When she encountered the edge of the leaf blister, she turned back into the egg mass and repeated the transit across the egg mass. She transited the egg mass several times before restricting her drumming to a single egg, at which point she oriented her body to the egg's long axis while slowly walking forward drumming the leaf surface. She continued to sweep the surface of the egg blister until she encountered the end of the egg at which point she reversed her course and continued her drumming and sweeping movements until she encountered the opposite end. She repeated the antennations and sweeping of the egg blister several times before halting near the centre of the egg and engaging her ovipositor (= DRILL, Figs 4 and 5). When she engaged her ovipositor on the leaf surface, she spread her legs widely and used the tarsal claws to grip the leaf surface. The drilling (= thrusting movements) resulted from the bending/flexing of her legs to insert the ovipositor through the leaf surface and into the egg beneath. Once the ovipositor was inserted into the egg, the female would move it within the egg briefly before beginning the 'bouncing' movement that marked the oviposition of an egg. Only one parasitoid egg was laid per host egg.

The brochosomes adhering to the parasitoid's body were acquired during antennation of a brochosome dusted egg mass. The brochosomes were contacted before the female encountered an egg mass because they were usually spread several millimetres beyond the edge of the egg mass blister (Fig. 1b). A female's antennation of such an egg mass was interrupted frequently with bouts of grooming (= GROOM, Figs 4 and 5) to remove the brochosomes adhering to her body (Fig. 3c,d). When a female

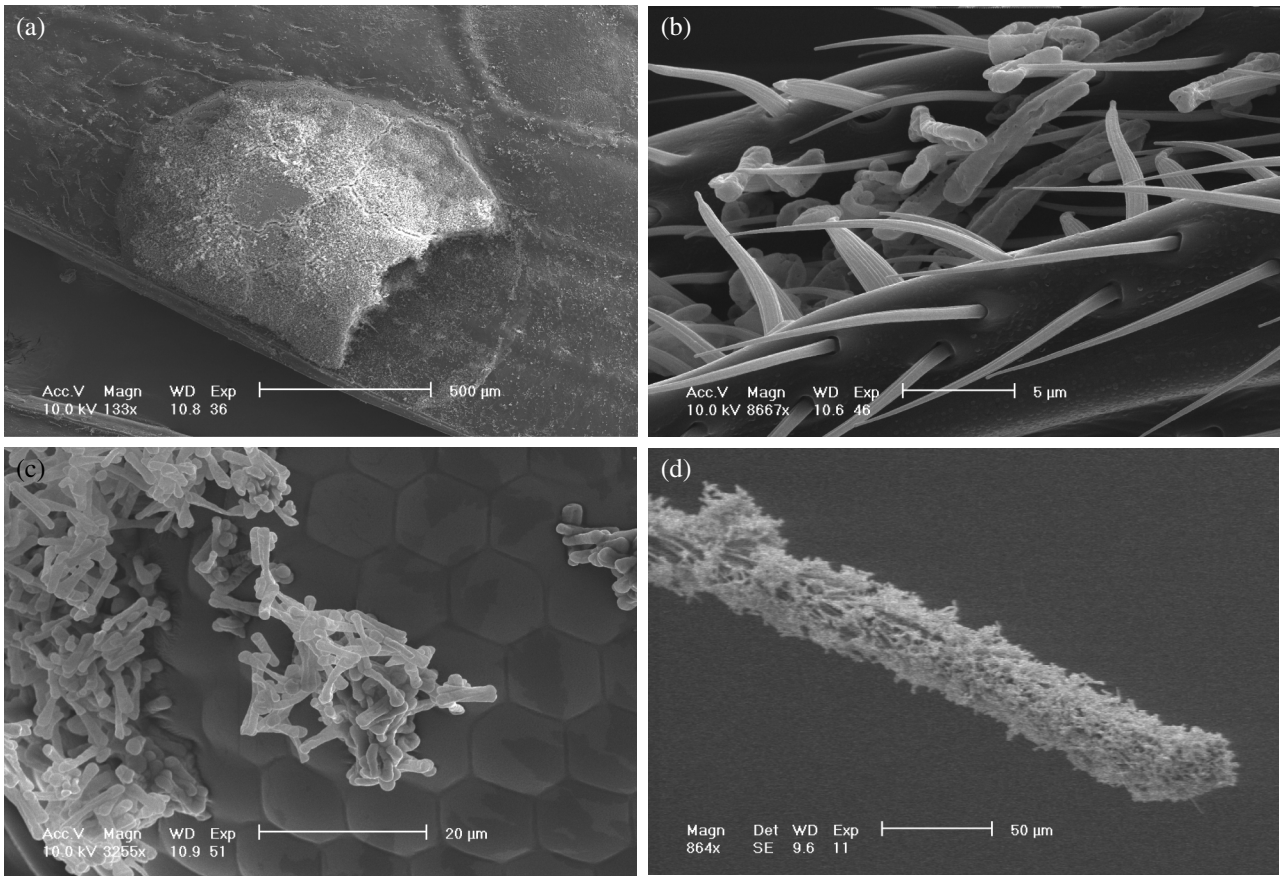

Fig. 3. Scanning electron microscope photographs illustrating how brochosomes can hinder oviposition by *Gonatocerus ashmeadi*. (a) Brochosome spot on *Homalodisca coagulata* forewing, (b) underside of *G. ashmeadi* antennal tip with brochosomes, (c) *G. ashmeadi* eye with brochosomes, and (d) i*G. ashmeadi* leg covered in brochosomes.

G. ashmeadi engaged her ovipositor on the leaf surface, she spread her legs widely and used the tarsal claws to grip the leaf surface in conjunction with the flexing of her legs (= thrusting movements) to insert the ovipositor. When debris or brochosomes clung to her tarsi, one or more legs would often fail to grip the leaf, which resulted in cessation of

		Brochosome-free Preceding behaviour						Brochosome-covered Preceding behaviour					
		WALK	ANT	DRILL	OVIP	GROOM	REST	WALK	ANT	DRILL	OVIP	GROOM	REST
Succeeding behaviour	WALK	0	34	32	4	35	22	0	57	33	1	121	18
	ANT	85	0	94	14	17	17	125	0	55	3	100	5
	DRILL	6	185	0	0	0	2	14	111	0	0	21	1
	OVIP	0	0	58	0	0	0	0	0	32	0	0	0
	GROOM	27	5	2	16	0	27	92	112	26	21	0	53
	REST	9	2	10	20	26	0	5	4	1	7	53	0

Fig. 4. Behavioural transition matrices as observed in behavioural experiments with *Gonatocerus ashmeadi* in the absence or presence of brochosomes on *Homalodisca coagulata* egg masses.

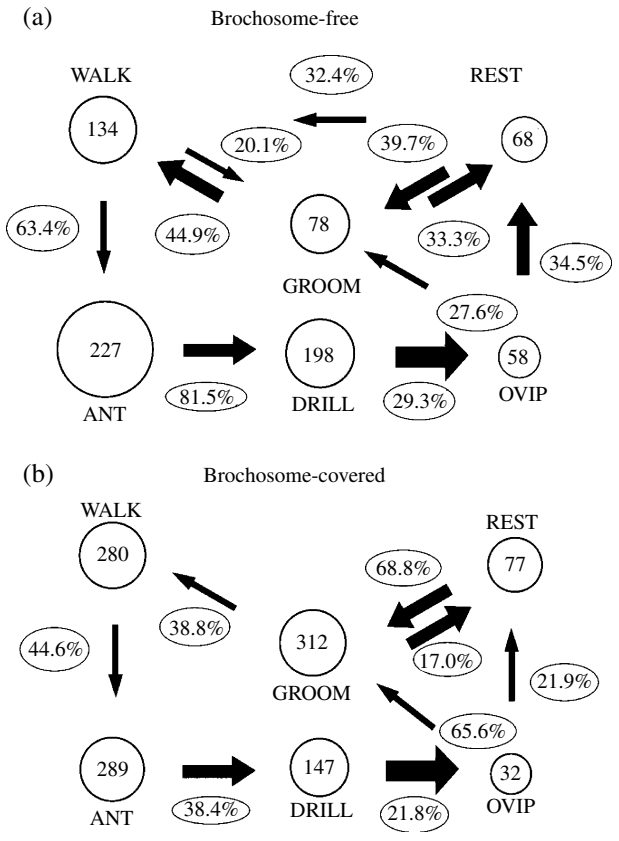

Fig. 5. Behavioural kinetograms for *Gonatocerus ashmeadi* examining *Homalodisca coagulata* brochosome-free (a) or brochosome-covered (b) egg masses. The areas of circles are proportional to the frequency of each behaviour started (frequencies shown). The arrows represent only those behavioural flows that occur significantly more often than expected by chance. The width of the arrows is proportional to the standardised residuals. The transitions are given as percentage of the occurrences.

drilling and the initiation of grooming and the re-initiation of the rapid antennation of the egg blister. Usually after one or more sweeps of the egg the female reinitiated her drilling by engaging the end of her ovipositor on the leaf surface near the centre of the egg. When the ovipositor successfully penetrated the leaf surface and entered the host egg, the female began her 'bouncing' movements. A successful oviposition is often followed by a bout of grooming.

A *G. ashmeadi* female may be able to avoid brochosomes on an egg mass by parasitising eggs through the upper leaf surface. Nineteen ovipositions were observed through the upper leaf surface into the eggs of a brochosome-covered egg mass by three *G. ashmeadi* females. In so doing, the female avoided encountering the brochosomes. Oviposition through the adaxial leaf surface accounted for 59% of ovipositions involving brochosome-covered egg masses.

Interruption trials

It was verified that the 'bouncing' movements exhibited by an ovipositing *G. ashmeadi* female indicated an oviposition. It was observed from one to three uninterrupted movements by nine females prior to interrupting the female's oviposition. This yielded 21 uninterrupted movements, 19 of which yielded an adult parasitoid, one yielded a dead parasitoid, and one yielded neither a parasitoid nor a *H. coagulata* nymph. In the nine cases in which a 'bouncing' female was interrupted, none of the interrupted ovipositions produced a parasitoid. Four of the nine eggs contained a dead, *H. coagulata* nymph; one egg yielded a live nymph; and four eggs failed to yield a parasitoid or nymph. Interruption showed that an egg was laid during the 'bouncing' movement by the parasitoid.

Behavioural transition matrices

The transitions between the six behaviours were observed in the frequencies shown in the two transition matrices (Fig. 4). Successful foraging bouts by female *G. ashmeadi* were characterised by behavioural sequences that consisted of walking while antennating on the leaf surface, antennating, drilling, and ovipositing within *H. coagulata* eggs. When foraging on leaves with *H. coagulata* egg masses heavily coated with brochosomes, female *G. ashmeadi* were more likely to interrupt this sequence of behaviours with bouts of grooming (Fig. 4a vs. 4b, ANT to DRILL). The relative number of transitions between these six behaviours differs significantly for parasitoids parasitising brochosome-free vs. brochosome-covered egg masses when comparing the full transition matrices ($G_{25} = 351.8$, $P << 0.001$; Haccou & Meelis, 1992). Thus the suite of behaviours displayed in the experiments with brochosome-free and brochosome-covered egg masses is significantly different.

Transitions between behaviours

A female *G. ashmeadi* that encountered and parasitised an *H. coagulata* egg mass either free or nearly free of brochosomes (Fig. 1a) manifested the behavioural sequence: walk, antennate, drill, and oviposition as the dominant behavioural sequence (Fig. 5b). Few bouts of grooming occurred once she had encountered an egg mass (Fig. 4, left). This contrasts with frequent bouts of grooming that interrupted the antennation of an egg mass or egg when the egg mass was heavily covered with brochosomes (Fig. 4, right). This often led to interruption of antennation by bouts of grooming. It should be noted that the transition from antennate to groom is not represented by an arrow in Fig. 5a,b. This is because, given the frequencies of behaviour, this transition does not positively deviate from random transitions: in the brochosome-free cases grooming occurred only 78 times, whereas this amounted to 312 in the brochosome-covered case.

Thus, the principal difference between the kinetograms for a parasitoid foraging on a brochosome-covered vs. one foraging on brochosome-free egg mass was the overall frequency of grooming. *Gonatocerus ashmeadi* females foraging on brochosomes-covered egg masses were much more likely to interrupt their ovipositional sequence with grooming bouts [larger circle in the centre of the brochosome-free (Fig. 5a) vs. brochosome-covered egg mass (Fig. 5b)].

Survival analysis

Table 1 displays the results of our proportional hazards analysis, where Z is the test statistic of interest. To be significant, Z must equal or exceed a $\chi^2_{5,\alpha=0.05} = 11.07$ because the model comprises five covariates. exp β is the hazard ratio and gives the magnitude of the covariate's effect if β differs significantly from zero. If the hazard ratio exceeds unity for a binary covariate, the hazard rate is greater at the value 1 than at the value 0. Consequently, the parasitoid is likely to fail sooner and the time to an event of interest will on average be shorter.

The analysis shows that in the presence of a brochosome-covered egg mass, female *G. ashmeadi* will antennate more often and in shorter bouts (Table 1, column 2 and 3 in rows ANT-int and ANT-B; Fig. 6a). She will also be less likely to drill (Fig. 6b) and more likely to groom (Fig. 6c) for longer periods (second and third column of Table 1). Irrespective of the presence or absence of brochosomes, an ovipositional experience increases a female's tendency to antennate and decreases her tendency to cease antennating. Thus antennating occurs more often and lasts longer after an ovipositional experience. Also once a parasitoid has oviposited in a host, she is more likely to drill subsequently and to take less time to do so. An ovipositional experience increases the probability per time unit to start another oviposition bout. However, it does not affect the duration of an oviposition. Larger egg masses lengthen the duration of an oviposition and older parasitoids antennate more frequently than younger ones. When foraging on the side of the leaf with an egg mass, a female will groom and antennate less often, and spend more time antennating and walking than a female foraging on the upper leaf surface, i.e. the leaf surface lacking the egg mass blister. Presumably the eggs within the egg mass on the underside of the leaf are much more difficult to detect and localise when searching the upper leaf surface. An example of a residual plot, given in Fig. 6d, is made for all fixed covariates. No deviations from proportionality were detected.

Discussion

These laboratory studies indicate that brochosomes may adversely affect *G. ashmeadi*'s parasitisation efficacy. Behavioural assays, survival analyses, and scanning electron microscopy all suggest that brochosomes have the potential to affect *G. ashmeadi*'s oviposition efficiency. When encountering a brochosome-covered egg mass, the female *G. ashmeadi* tended to increase her antennating frequency. This increased frequency was associated with individual shorter periods of antennation, interrupted by more frequent periods of intensive grooming, than was the case with a female encountering an egg mass lacking brochosomes. These behaviours were also associated with a decreased tendency to drill into an egg in a brochosome-covered egg mass than was the case with the eggs of a brochosome-free egg mass. Moreover, after antennating a brochosome-covered egg mass, a *G. ashmeadi* female had brochosomes present on her antennal sensory hairs which were associated with periods of intensive grooming. It is suspected that the brochosomes interfered with the female's

Table 1. A summary of Cox's regression results. Behavioural durations and intervals (in seconds) are sorted by rows, covariates are ordered in columns with influence (expβ) and test statistic (Z) with 5 degrees of freedom.

	BROCH PRESENT		LEAF SIDE		OVIP		EMS		WASP AGE	
	expβ	Z	expβ	Z	expβ	Z	expβ	*Z*	expβ	Z
WALK-int[a]	1.158	1.10	1.336	4.95	0.890	3.64	1.146	0.90	0.851	1.07
WALK-B[b]	1.003	0.00	0.623	20.29****	1.243	7.82	0.988	0.01	0.923	0.34
ANT-int	1.318	10.89**	0.541	47.94*****	1.217	25.36*****	0.867	2.86	1.374	11.10***
ANT-B	1.921	25.59*****	0.633	10.80**	0.804	13.49***	0.904	0.62	1.175	1.51
DRILL-int	0.466	21.51*****	0.956	0.08	1.637	40.15*****	0.596	9.33*	1.240	1.83
DRILL-B	0.785	1.92	1.146	0.50	1.725	36.77*****	0.851	0.92	0.903	0.40
OVIP-int	0.589	3.27	0.397	6.24	1.691	11.34***	0.553	4.04	0.929	0.06
OVIP-B	1.699	2.68	0.226	7.83	1.406	4.38	0.222	13.35***	0.598	2.94
GROOM-int	2.638	39.84*****	0.638	12.18***	0.961	0.47	0.991	0.00	0.659	8.87
GROOM-B	0.639	9.57*	0.921	0.52	1.023	0.12	0.934	0.26	1.400	4.98
REST-int	0.814	1.06	1.283	1.52	1.287	6.59	0.815	0.87	0.831	0.70
REST-B	1.047	0.04	0.634	4.54	0.927	0.39	1.101	0.20	0.617	4.76

*$P < 0.1$, **$P < 0.06$, ***$P < 0.05$, ****$P < 0.01$, *****$P < 0.001$.

[a]int, the interval between two subsequent occurrences of this behaviour.

[b]B, the duration of the behaviour.

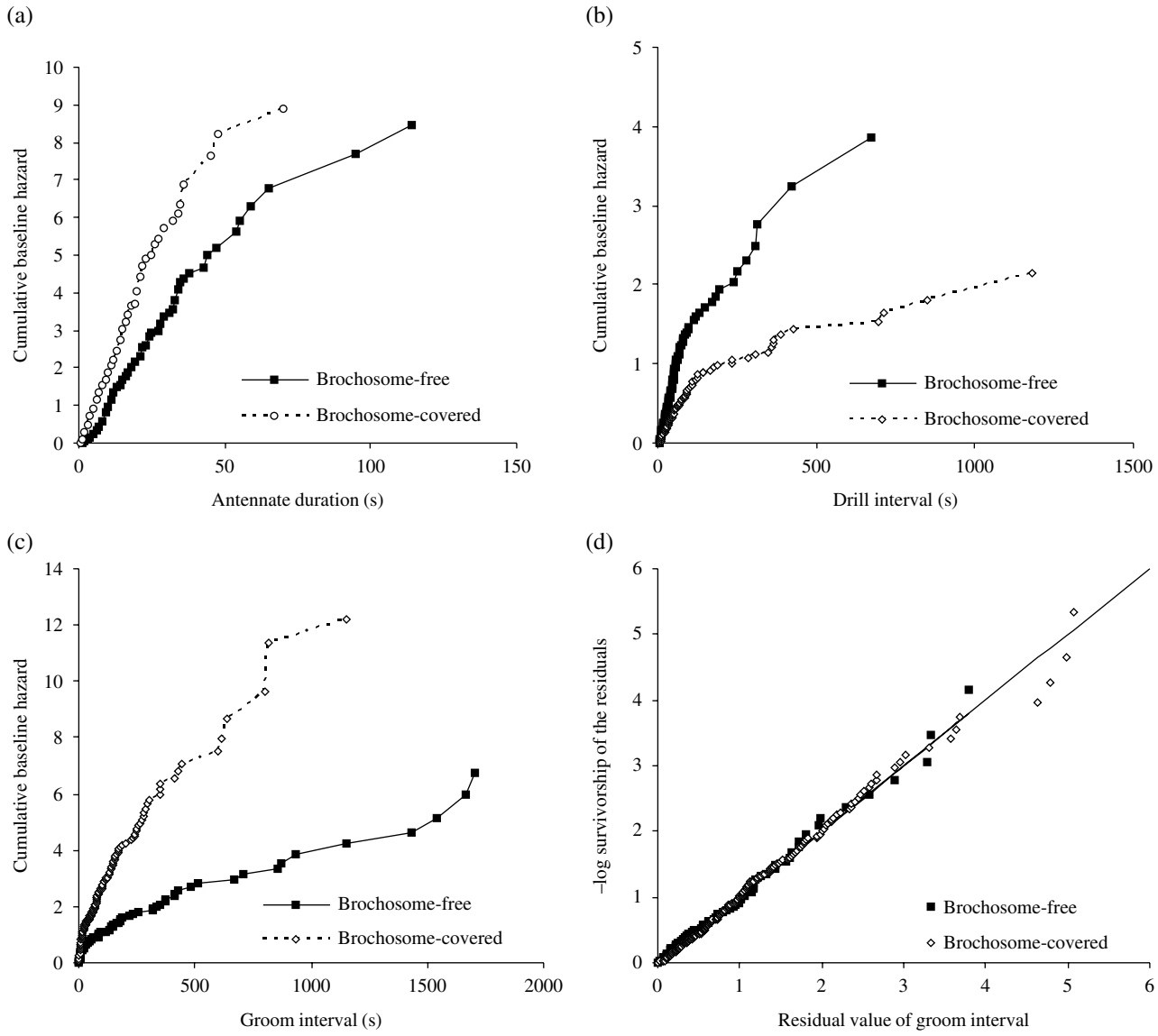

Fig. 6. (a) The effect that brochosome presence has on the length of antennation periods, (b) the effect that brochosome presence has on the interval time between drilling periods, (c) the effect that brochosome presence has on the time between grooming periods, and (d) a residual plot as check of the proportionality assumption.

ability to detect and/or locate the eggs within a brochosome-covered egg mass. As antennation precedes drilling, this suggests that brochosomes hinder *G. ashmeadi*'s oviposition success.

With ovipositional experience, a *G. ashmeadi* female was more likely to begin longer periods of antennation with an increased likelihood that a drilling and ovipositional event ensued. If a female had at least one ovipositional experience, more successful ovipositions were likely to follow involving shorter intervals between ovipositions. However, the presence of brochosomes resulted in longer intervals between drilling events and the female was more likely to stop drilling after the first oviposition. Additionally, in some instances when brochosomes were present, parasitoid movements became slower, and occasionally the parasitoid laid down on the leaf (12% of cases).

Grooming is the behaviour with the greatest effect (exp β) of all of the six behaviours. In the presence of brochosomes, grooming is less likely to cease (i.e. a lower hazard rate) than that observed by a female encountering a brochosome-free egg mass ($P < 0.1$). When taken together, the increased antennation frequency manifested for shorter periods intermixed with frequent periods of grooming associated with the brochosome-covered *H. coagulata* egg masses suggests that brochosomes significantly hindered parasitisation rates of *H. coagulata* eggs. Whether this

results in a reduced percentage parasitism of eggs in brochosome-covered egg masses remains to be field tested.

As mentioned above, a *G. ashmeadi* female may be able to avoid brochosomes on an egg mass by parasitising eggs through the upper leaf surface. This illustrates the behavioural adaptability of this parasitoid to unfavourable conditions, because females did not display such behaviours on brochosome-free egg masses. Similar oviposition behaviours have been observed in *Entedononecremnus krauteri* Zolnerowich and Rose (Hymenoptera: Eulophidae), a parasitoid of the giant whitefly, *Aleurodicus dugesii* Cockrell (Homoptera: Aleyrodidae) which parasitises whitefly nymphs through the upper leaf surface. Giant whitefly nymphs produce copious wax filaments that extend vertically from the dorsal surface and are thought to interfere with a parasitoid's access to the whitefly nymphs if it were to attempt parasitisation directly into the nymph (Bellows *et al.*, 2002).

Scanning electron micrographs clearly illustrate the presence of many brochosomes on *G. ashmeadi* and this material readily accumulated and adhered to various body parts, including the tarsi, antennae, and compound eyes. Observations indicated that the parasitoids attempted to remove these brochosomes by grooming the antennae, ovipositor, body, and wings with its legs. During these grooming periods, large clumps of brochosomes were removed from these appendages but accumulated on the tarsi. The preening subsequently moved them to the prothoracic tarsi. Brochosomes adhering to fore tarsi were then removed with the mandibles and they ultimately ended up on the parasitoid's frons. The consequence of this accumulation is uncertain.

Brochosomes covering an egg mass adversely affected antennation, increased the interval between drilling events, and increased the time allocated to grooming by a parasitoid. Whether such effects manifest themselves under field conditions is uncertain, as these parasitoids may not be time limited and multiple parasitoids may make multiple visits to egg masses bearing brochosomes. Further, it is unknown what proportion of *H. coagulata* egg masses have sufficient brochosome covering under field conditions to impede parasitism, and how varying levels of brochosome density affect a parasitoid's success. These issues need to be investigated.

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