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Egg mass structure of the processionary caterpillar *Ochrogaster lunifer* (Lepidoptera: Notodontidae): is the outer egg layer sacrificed for attack by the egg parasitoid *Anastatus fuligispina* (Hymenoptera: Chalcidoidea: Eupelmidae)?

Mizuki Uemura,[1,2*,†] Lynda Perkins,[1] Andrea Battisti[2] and Myron Zalucki[1]

[1]*School of Biological Sciences, The University of Queensland, St Lucia, QLD Australia.*
[2]*Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Padova, Italy.*

Abstract Each life stage of an insect faces the challenge of various mortality factors. Through experimental and observational studies, we use those mortality agents to our advantage to control outbreaks of pest insects. The processionary caterpillar *Ochrogaster lunifer* Herrich-Schäffer, 1855, is a widespread native moth in Australia that defoliates host trees and causes medical problems in humans and animals. *Anastatus fuligispina* (Girault 1939) is an egg parasitoid described from eggs of *O. lunifer* in eastern Australia nearly 80 years ago for which few life history traits are known. This is the first study to investigate the life history of *A. fuligispina*, factors associated with parasitism levels in *O. lunifer* egg masses and its impacts on egg mortality. We found that parasitism level was related to the total number of eggs in an *O. lunifer* egg mass, with higher parasitism occurring in masses with fewer eggs. The inaccessible physical structure of the *O. lunifer* egg mass by layering and encasing eggs with other eggs and the searching efficiency of the parasitoid are possible key factors. Other variables such as exposure time in the field, host tree species and number of undeveloped eggs in the egg mass did not affect the level of parasitism. Further investigations on the life history of *A. fuligispina* may open possibilities for its application in controlling *O. lunifer* populations.

Key words defence against parasitism, host–parasitoid relationship, natural enemy, Queensland.

INTRODUCTION

Processionary caterpillars of the moth *Ochrogaster lunifer* Herrich-Schäffer are common and widespread across coastal and inland Australia (Floater 1996). Populations of *O. lunifer* vary in morphology, host tree, oviposition location and larval nesting behaviour, which suggests the existence of two or more species (Floater 1996). Therefore, *O. lunifer* is described by multiple common names; the common name processionary caterpillars will be used here for consistency with Floater's (1996) work. All information in this paper refers to *O. lunifer* of the ground nesting form that feeds exclusively on *Acacia* species. In outbreak years, this species defoliates host trees and establishes new colonies where hosts are present (Floater & Zalucki 1999). This species causes significant medical problems for humans and animals because the caterpillars have urticating hairs called true setae (Battisti *et al*. 2011; Perkins *et al*. 2016). Medical problems include urticaria and allergic reactions in humans, tongue necrosis in dogs (Battisti *et al*. 2011) and miscarriages in horses (Cawdell-Smith *et al*. 2012).

Ochrogaster lunifer has a univoltine lifecycle, consisting of eight larval instars from December to May, pre-pupa from May to September, pupa from September to November and adult moth from October to November in Queensland (Floater 1996). Females deposit 150–550 eggs at the base of a host tree trunk and cover them with flat and filamentous scales from their anal tuft, creating a circular mass 25–35 mm in diameter and 10–15 mm deep (Floater & Zalucki 1999). These scales are urticating (Perkins *et al*. 2016) and provide protection from natural enemies (Floater & Zalucki 1999). The urticarial nature of both the larvae and adults makes their physical removal for pest control difficult and creates an occupational health and safety risk. However, natural mortality agents, such as predators and parasitoids, can be used to reduce *O. lunifer* populations significantly (Floater 1996).

The most vulnerable life stages of *O. lunifer* are the egg and first instar larvae (Floater 1996). Common causes of mortality include egg parasitism by chalcid wasps, egg and first instar predation by dermestid beetles and larval parasitism by tachinid flies (Floater 1996). The eggs of *O. lunifer* are parasitised almost exclusively by *Anastatus fuligispina* (Girault) (Floater 1996). Dermestid species with larvae that eat *O. lunifer* eggs and first instar larvae include *Dermestes ater* DeGeer and *Trogoderma apicipenne* Reitter (Floater 1996).

Investigating mortality rates of *O. lunifer* is essential to understanding their population dynamics. Floater and Zalucki (1999) concluded that egg and larval parasitism was a minor mortality agent and dermestid predation was the dominant mortality factor in the populations they studied in south-east Queensland.

*mizuki.uemura@studenti.unipd.it

†Present address: Viale dell'Università, 16, 35020 Legnaro PD, Italy.

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However, they collected egg masses that had failed to develop in January, which is at least a month after the last expected hatching of *O. lunifer* neonates. As development time from egg to first instar is 3–4 weeks, and last oviposition is approximately November (Floater 1996), successfully parasitised eggs may not have been evident at the time of their study, and so underestimated.

Although egg parasitoids are common biological control agents for insects, those species associated with emerging pests are still poorly known. Our aims were to (1) determine the levels of parasitism of *O. lunifer* egg masses collected near the time of oviposition and (2) describe aspects of the life history for *A. fuligispina*. This information will contribute to the ecological and biological characterisation of *A. fuligispina* as a biological control agent against *O. lunifer*. Targeting *O. lunifer* in the early stages is an effective way to manage the species, because an initial reduction in the cohort will make the cohort more vulnerable to extinction and limit the damage caused prior to cohort extinction (Floater & Zalucki 1999).

MATERIALS AND METHODS

Egg mass collection and preservation

Ochrogaster lunifer egg masses were collected from The University of Queensland, Gatton Campus, Queensland, Australia (−27°56′S, 152°34′E). Eight locations within the campus where various *Acacia* species occur were monitored weekly for new egg masses. Host trees included *Acacia aneura*, *Acacia concurrens*, *Acacia fimbriata*, *Acacia podalyriifolia* and *Acacia salicina*. Non-host trees within the eight locations were also monitored for egg masses. Egg masses were collected at initial sighting, while others were marked and collected 1, 2 and 4 weeks later. The first egg masses laid in the season were observed and collected on the 5th of October 2017, and the last were observed and collected on the 16th and 23rd of November 2017, respectively, with a total collection of 65 egg masses.

Egg masses were removed from the trunk using a wood chisel, placed in a 120 mL jar and covered with fine mesh. Jars were kept in the laboratory for 28 days at 25 °C and exposed to the same photoperiod as the external environment. Upon collection, *A. fuligispina* were found within some egg masses; these wasps were not counted in the analyses. Egg masses were checked daily and any emerged *O. lunifer* and *A. fuligispina* recorded. If parasitoids emerged, a droplet of honey was supplied on the mesh for food. After 28 days, jars with egg masses were placed in a −20 °C freezer overnight to terminate development. Twenty-eight days was chosen because parasitoids and *O. lunifer* larvae hatch between 2.5 to 4 weeks (see Statistical analyses section, Floater 1996). Each egg mass was transferred into a 50 mL Falcon tube with 20 mL of 70% ethanol to fix the eggs and help contain the urticating scales before processing.

Egg fate

Contents of the Falcon tube were poured into a 100 × 15 mm Petri dish and were examined under an Olympus SZX16 stereomicroscope. Featherweight forceps were used to separate scales and debris from hatched and unhatched eggs, *O. lunifer* larvae, *A. fuligispina* wasps and dermestid larvae. Contents of hatched and unhatched eggs were determined using morphological examination (see below) and counted. Measurements of *A. fuligispina* adults were taken using Olympus DP26 digital camera software.

Morphological examinations

Initially, parasitised and unparasitised eggs were distinguished following Floater and Zalucki (1999). From our findings, detailed descriptions of developing parasitised and unparasitised eggs were determined and reported in the Results section. Adult *A. fuligispina* were examined to differentiate the characteristics of the sexes.

Statistical analyses

Parasitism level was determined by the formula:

$$\frac{(\text{Parasitised eggs} - \text{Emerged } A.\textit{fuligispina} \text{ wasps})}{\text{Total number of eggs}}.$$

Parasitised eggs refer to the total number of hatched and unhatched *A. fuligispina* eggs (see Morphological examinations section in the Results section for description) in an egg mass.

Emerged *A. fuligispina* wasps refer to the total number of wasps that emerged from parasitised eggs.

Total number of eggs refers to the total number of hatched and unhatched *A. fuligispina* and *O. lunifer* (including undeveloped) eggs, giving a total count of eggs deposited by the *O. lunifer* female.

A generalised linear model (GLM) was used to determine if parasitism was associated with duration of exposure (<7 or >7 days), host tree species, the number of undeveloped eggs or the total number of eggs (unhatched and hatched) in the egg mass using statistical software RStudio version 1.1.419 (RStudio, Inc. 2016) (see Codes S1 and S2 for R codes used for full and reduced GLM models, respectively). Akaike information criterion (AIC) values for all possible models were compared to determine the best model to explain the data. Egg masses collected a week or more after the initial sighting (>7 days) were grouped for analyses due to the uneven sample size at each time interval. Before pooling, a GLM Gaussian distribution (Faraway 2016) was performed and showed no significant difference in parasitism levels between egg masses collected a week and more than a week after initial sighting ($P > 0.5$). An alpha value of $P < 0.05$ was taken as statistically significant.

An approximate development time of *A. fuligispina* was calculated as the period between the dates of collection at initial sighting (from egg masses <7 days) to the dates of parasitoid emergence. Parasitism level and total number of eggs in an egg mass were modelled against the number of dermestid larvae found in the egg mass to determine if there were any effects (Codes S3 and S4, respectively).

RESULTS

Egg fate

The number of eggs in each egg mass ranged from 67 to 479 with an average of 301 (SE ± 12 eggs, N = 65 egg masses). Sixty-two egg masses (95.4%) were parasitised by *A. fuligispina*, and an average of 38.4% (SE ± 2.9%, range 1.2–90.2%) of eggs was parasitised in each egg mass (Fig. S2). One egg mass without parasitism was found on each of the following hosts: *A. concurrens*, *A. salicina* and the non-host species *Flindersia xanthoxyla*. The sex ratio of emerged *A. fuligispina* was 3.4 females to one male (N = 142). Average (approximate) development time for *A. fuligispina* from egg mass collection to adult emergence was 22.5 days, range 19 to 26 days (SE ± 2 days, N = 17).

The model of best fit according to the lowest AIC value was as follows: Parasitism within egg masses ~ Total number of eggs in the egg mass (Codes S1 and S2). Parasitism was significantly greater in egg masses with fewer eggs (GLM: $t = -3.425$, $N = 65$, $P < 0.01$; Fig. 1). There were no significant effects of exposure duration (Fig. S2), host tree species (Fig. S3) or number of undeveloped eggs (all $P > 0.1$; Code S1) on the proportion of eggs parasitised.

Twenty-two *Trogoderma* sp. larvae were found in 10 egg masses, which is 15% of the total egg masses analysed. Occurrence of dermestid larva in an egg mass ranged from one to four individuals and had no influence on observed parasitism level (GLM: $t = 1.037$, $N = 65$, 0.304) or was not related to total number of eggs in an egg mass (GLM: $t = -1.737$, $N = 65$, 0.0873).

Morphological examinations

The fate of eggs were categorised into three groups (Fig. S1):

Normal with Ochrogaster lunifer development

Newly laid *O. lunifer* eggs appear white and opaque (Fig. S1, 1A–F). After 2 weeks, a white segmented embryo with mandibles and eyes become visible. Seven days later, the head capsule and rest of the embryo body becomes pigmented. Upon hatching, the larva leaves a large jagged exit hole, and the empty egg shell is transparent.

Parasitised with Anastatus fuligispina development

Three to 4 days after *A. fuligispina* parasitises an *O. lunifer* egg, the host egg appears speckled, with specks increasing through time (Fig. S1, 2A–F). The specks are dark brown and cover the internal surface of the egg shell. Six to 9 days after parasitisation, the body form of the parasitoid larva becomes prominent, yellow and half-moon-shaped. The larva enters a pre-pupal stage, and the dark eye pigments (ommochromes) become visible (Quicke 2015) 12 to 15 days post-parasitisation. Melanisation of the cuticle is evident as the pupa darkens from transparent grey to black, before an adult wasp emerges from the egg. The wasp leaves a neat round exit hole, and the empty egg shell is speckled with the meconium remaining inside. Female and male wasps can be distinguished by the presence/absence of the ovipositor, respectively, wing pattern and leg colouration. Females have an orange-coloured ovipositor with an average length of 1.59×10^{-1} mm (SE ± 1.27×10^{-2} mm, N = 8), which is approximately 1/14th of the body length. Forewings of the female are elongated and have thick dark brown horizontal bands across the basal, discal and submarginal to marginal areas. Male forewings are completely transparent and rounded in shape. Legs of females are uniform in colour, and males have cream-coloured basitibial plate and basitarsus. Males are smaller than females, with an average body length of 1.69 mm (SE ± 1.8×10^{-2} mm, N = 37) compared to females that are 2.14 mm (SE ± 2.8×10^{-2} mm, N = 36).

Undeveloped eggs

Morphology of undeveloped *O. lunifer* eggs vary, and the reason for their failure is unknown (Fig. S1, 3A–D). The eggs look neither normal nor parasitised. Such eggs have no consistent distinguishable shape and may be variable in colour, dry or contain an air bubble. Examples include eggs that have a condensed yolk

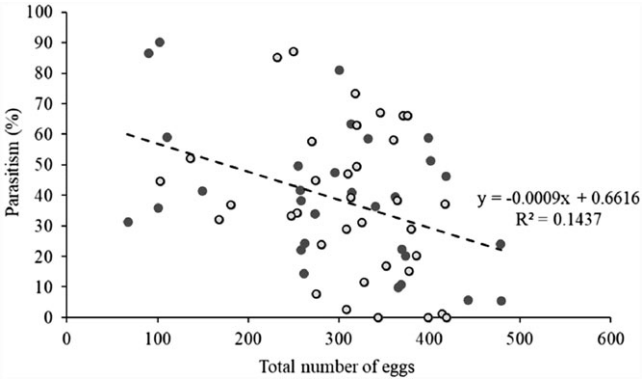

Fig. 1. Relationship between egg parasitism by *Anastatus fuligispina* and total number of eggs in an *Ochrogaster lunifer* egg mass. Each point represents parasitism (%) by *A. fuligispina* of each *O. lunifer* egg mass collected from The University of Queensland, Gatton Campus, Queensland, Australia. Light grey points with an outline and dark grey points without an outline are egg masses collected <7 and >7 days, respectively. The total number of eggs ranged from 67 to 479 eggs in 65 egg masses analysed. Parasitism ranged from 0% to 90.2% in an egg mass.

with air surrounding it and/or dry looking, appear donut-like or have dark pigmentation without any specks on the eggshell.

DISCUSSION

The natural mortality of *O. lunifer* has been documented (Floater & Zalucki 1999) at a regional population level; however, the parasitism level within egg masses and factors associated with parasitism were unexplored. Our study determined levels of parasitism by *A. fuligispina* at the population and egg mass level and examined possible variables associated with parasitism: the duration of exposure, host tree species, and the numbers of undeveloped eggs and total eggs in an egg mass.

Levels of parasitism differed with total number of eggs in an egg mass. Parasitism was higher in *O. lunifer* egg masses with fewer eggs, and we propose that the structure of an *O. lunifer* egg mass may explain this. Female *O. lunifer* moths deposit eggs in a rough pyramid shape, with eggs in the base layer on the trunk glued and encased by outer layers of eggs (Floater 1998). Ovipositor length of *A. fuligispina* is 1.59×10^{-1} mm (SE ± 1.27×10^{-2} mm), and *O. lunifer* egg diameter is 1.33 mm (SE ± 5.57×10^{-3} mm, $N = 261$ eggs, unpublished data). Therefore, once the whole *O. lunifer* egg mass is laid, *A. fuligispina* may only be able to parasitise the outer layer of eggs. Low level of parasitism was demonstrated in *Euproctis chrysorrhoea* (L.) (Lepidoptera: Lymantridae), that also lays egg masses covered with tuft scales, when only the outer layer was attacked by the egg parasitoid *Telenomus turkarkandas* (Sz.) (Hymenoptera: Scelionidae) (Bin *et al*. 1988). Their findings correlate with our data and explain why there was less parasitism in *O. lunifer* egg masses with more eggs; because with each additional layer of eggs, proportionally fewer are within of reach of *A. fuligispina*. Bin *et al*. (1988) also demonstrated that *T. turkarkandas* successfully parasitised eggs of *E. chrysorrhoea* in the base layer. For parasitoids to access the base layer and other layers of eggs, they must be present during the oviposition of the female moth (Bin *et al*. 1988). For this to be possible, some parasitoids have a phoretic relationship with their host (Arakaki 1990; Bin *et al*. 1988). *Telenomus* sp., egg parasitoid of *Euproctis taiwana*, were observed in the anal tuft of female moths (Arakaki 1990). At the time of first oviposition by the *E. taiwana* female, parasitoids left the female's body and parasitised the eggs (Arakaki 1990).

Parasitism did not vary with duration of host exposure, suggesting that *A. fuligispina* parasitise *O. lunifer* eggs soon after they are laid (Fig. S2). Parasitoids are generally active and search for host eggs during the day (Arakaki *et al*. 2011). However, some hymenopteran parasitoids have found a way to overcome this problem (Fatouros & Huigens 2012). In the field at night, *A. fuligispina* was observed creeping in the egg mass as soon as the *O. lunifer* female started ovipositing (M. Uemura 2018, personal observation, Video S1). The egg mass was collected early next morning and was later confirmed that *A. fuligispina* emerged from the eggs. *O. lunifer* females can sustain flight for prolonged periods (20 min and possibly more) (Floater 1996). However, *Anastatus* spp. adults are weak flyers (R. Llewellyn pers. comm. 2018), which means it will be difficult for *A. fuligispina* to follow an *O. lunifer* female in flight. Therefore, this observation of *A. fuligispina* present at the site of oviposition may suggest that *A. fuligispina* either has a phoretic relationship with *O. lunifer* or the eggs have specific kairomones. A phoretic relationship is indirectly suggested by Girault's (1939) description of *A. fuligispina*, where he mentions that the parasitoids were reared from three female *O. lunifer* moths. *A. fuligispina* may locate *O. lunifer* females from the pupal stage or from pheromones when the female is calling and crawl into her anal tuft, which is observed in other parasitoids (see Arakaki *et al*. 2011). An adaptation to parasitise soon after host oviposition may have developed in *A. fuligispina* to maximise their fecundity in *O. lunifer* eggs that will later be inaccessible (i.e. outer eggs will encase inner eggs in the egg mass). There is also evidence that the survival of parasitoid larva declines as host eggs get older (Boivin 2010), indicating that earlier oviposition maximises fitness of the offspring.

Host tree species had no influence on parasitism. This suggests that *A. fuligispina* can detect *O. lunifer* female moths and/or parasitise egg masses with the same efficiency on any *Acacia* host tree (Fig. S3). Only one *O. lunifer* egg mass was found on a non-host species, *F. xanthoxyla*. This tree was surrounded by *A. fimbriata*, and the egg mass was not parasitised. Hymenopteran parasitoids accurately find hosts by using olfactory semiochemicals produced by hosts, visual signals from the colour contrast of the host from the plant surface and contact stimuli of the host's physical defence (Xiaoyi & Zhongqi 2008). Thus, it may be possible that *A. fuligispina* use host tree semiochemicals additional to volatiles produced from *O. lunifer* as an olfactory cue to an egg mass and/or female moth, and the egg mass laid on *F. xanthoxyla* may have been undetectable. However, more data are required to confirm this.

The undeveloped eggs in an *O. lunifer* egg mass were considered as a variable that may affect parasitism, because other *Anastatus* spp. can develop in sterile eggs (R. Llewellyn pers. comm. 2018). There was no significant difference in parasitism with number of undeveloped eggs, which suggests that *A. fuligispina* can parasitise infertile/failed *O. lunifer* eggs as observed for other species. Occurrence of *Trogoderma* sp. larvae in egg masses suggests that some eggs may have been consumed regardless of whether the eggs were parasitised or not. However, in our analyses, the number of parasitised eggs and total number of eggs in an egg mass did not differ between egg masses with and without *Trogoderma* sp.

Floater (1996) found predation by the dermestid larvae *D. ater* and *T. apicipenne*, on the egg and first instar larvae as the most important cause of mortality for the early stages of *O. lunifer*. Occurrence of at least one dermestid larva in an egg mass accounted for 30–40% of egg masses surveyed from mainland south-east Queensland and North Stradbroke Island (Floater 1996), which is double our observations (15%) from Gatton. Floater (1996) also stated that egg parasitism was uncommon and occurrence of *A. fuligispina* accounted for 1.2–3.6% of egg masses from two study sites, whereas in our study at Gatton, 95% of egg masses were parasitised by *A. fuligispina*. The very high parasitism levels may reflect a build-up of parasitoids in the area as

O. lunifer has been abundant at Gatton for some time. However, it is unclear what methodologies and how many egg masses Floater (1996) analysed to determine egg predation and parasitism. Additionally, egg masses may have been analysed after *A. fuligispina* emergence and empty parasitised eggs may have been disregarded in the count. Predation and parasitism in *O. lunifer* can be variable within populations (Floater & Zalucki 1999).

Tsankov *et al*. (1996) stated that hymenopteran egg parasitoids can parasitise host embryos at older stages of development and eggs from the same egg batch from which they emerged. More experiments and microscopic analyses are required to confirm if *A. fuligispina* can parasitise late developing *O. lunifer* embryos. Upon *O. lunifer* egg mass collection, each egg mass was confined in a single container for 28 days in the lab. *A. fuligispina* develops in 19–26 days; therefore, females that emerged from egg masses collected when it was 0 to 14 days old could possibly oviposit fertilised/unfertilised eggs into the same egg mass. Our studies confirmed that the parasitism level within egg masses was not significantly different between egg masses collected <7 vs. >7 days. Therefore, it may be assumed that emerged *A. fuligispina* females did not parasitise any eggs because *O. lunifer* eggs were older and/or the structure of the egg mass prevented further parasitism.

Arthropod predators of Lepidoptera are diverse and tend to be generalists (Floater & Zalucki 1999). However, with urticating scales covering *O. lunifer* egg masses, only specialised natural enemies may have adaptations to overcome this defence. Nearly 80 years after the discovery of *A. fuligispina* by Girault (1939), no hosts other than *O. lunifer* have been recorded (Floater 1996), suggesting a very close host–parasitoid association. It is possible that *A. fuligispina* females emerging from host eggs early in the season parasitise other *O. lunifer* egg masses, resulting in two or more generations during the 2 months of *O. lunifer* oviposition. However, after *O. lunifer* oviposition ends, *A. fuligispina* must either reproduce in other hosts or undertake a 10 month diapause as described in other parasitoids (see Schmidt *et al*. 1999).

There are several important gaps in our understanding of the biology and life history of *A. fuligispina*. We need to confirm if *A. fuligispina* is phoretic (Girault 1939), by capturing adult *O. lunifer* moths of both sexes and checking them for the presence of any *A. fuligispina*. If *A. fuligispina* are phoretic, it is expected to find only female *A. fuligispina* present on the body, more specifically tuft scales, of female *O. lunifer*. Behavioural observations of *A. fuligispina* oviposition and counting how many *O. lunifer* eggs a single *A. fuligispina* female can parasitise should be determined. An experiment to confirm that *A. fuligispina* only parasitise outer eggs of the egg mass may be done by dissolving the glue attaching the eggs with a solvent and then removing and comparing the parasitism of outer eggs to the unexposed eggs underneath. Conducting a Y-tube olfactometer experiment using egg masses with and without host plant stimuli will be beneficial to understand if host plant chemicals are a cue for *A. fuligispina* to detect hosts. For effective pest management, bionomic data for the pest, its host plants and natural enemies are essential (Schmidt *et al*. 1999). Such investigations may build on the baseline data on the parasitism of *A. fuligispina* and may open possibilities for its application in controlling *O. lunifer* populations.

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SUPPORTING INFORMATION

Additional supporting information may/can be found online in the supporting information tab for this article.

Figure S1 1A – F Normal development of *Ochrogaster lunifer* larvae starting from 1A which is newly laid to 1F where a neonate larva has emerged. 2A – F Parasitised egg development of *Anastatus fuligispina* starting from 2A which is newly parasitised O. lunifer egg to 2F where a female wasp has emerged. 3A – D Undeveloped O. lunifer eggs of various unknown mortalities. The scale bar represents 1 mm for all images except for 1F^ and 2F^, which are 2 mm. Images taken by Mizuki Uemura using Olympus SZX16 stereomicroscope with DP26 digital camera attachment.

Figure S2 Average percentage (± SE) of eggs in each morphological category (normal, undeveloped and parasitised) of 65 *Ochrogaster lunifer* egg masses exposed to <7 days and >7 days (until 28 days) in the field. Number of egg masses analysed for each exposure duration is given above the bars.

Figure S3 The average percentage of eggs categorised as normal, undeveloped and parasitised following morphological assessment of 65 *Ochrogaster lunifer* egg masses collected from different host plants. Number of egg masses analysed for each host plant is given above the bars.

Code S1 R code used in the full Generalised Liner Model of parasitism level within egg masses as a function of all variables: exposure time, host tree species, the number of undeveloped eggs, and the total number of eggs.

Code S2 R code used in the reduced GLM using parasitism level as a function of total number of eggs.

Code S3 R code used in the GLM using parasitism level as a function of number of dermestids in the egg mass.

Code S4 R code used in the GLM using total number of eggs as a function of number of dermestids in the egg mass.

Video S1 *Ochrogaster lunifer* female ovipositing on *Acacia concurrens* at Gatton, Queensland. Various insects were flying around and onto the female moth. An *Anastatus fuligispina* was observed creeping in the egg mass left of the anal tuft. The O. lunifer female was dusted with orange fluorescent dust and shone with a UV torch. The video was recorded by Mizuki Uemura with an Apple iPhone 8 and speed of the video was slowed down 2x.