



## ORIGINAL ARTICLE

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# Circadian and seasonal flight activity differences between the sexes of the biocontrol agent *Eadya daenerys* (Hymenoptera: Braconidae) and the impact of host size on adult emergence

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## Abstract

Since its accidental introduction into New Zealand in 1916, the invasive eucalypt leaf beetle *Paropsis charybdis* remains a pest of economic significance to *Eucalyptus* forestry. For this reason, the braconid parasitoid *Eadya daenerys* has been approved for release as a classical biological control agent. To aid in field monitoring using hand netting of biocontrol releases and laboratory rearing protocols, the flight activity over 6 a year field research program of *E. daenerys* and the relationships between the parasitoid and the size of its beetle hosts were evaluated in the country of origin. Wasps were observed flying over 32 days from mid-November to mid-December in Tasmania. *E. daenerys* was found to exhibit a morning-based scramble competition mating system. Females flying in the early morning had lower mature egg loads ( $134.2 \pm 27.6$ ) than at other times of the day. In the first half of the season, male flight was most often seen in the early morning, whereas in the second half of the season, there was a 4.5-fold decrease in any wasps flying. Female flight activity significantly increased to 70% female in the evening hours from 5:00 PM to 8:00 PM, which by the second half of the season was almost exclusively female. Wasps were caught flying across a wide range of air temperatures (10.7–23.8°C), humidity and light levels, but even light winds reduced flight when average wind speed exceeded 0.27 m/s for males and 0.73 m/s for females. Beetle prepupal weight was a predictor of *E. daenerys* parasitism success with mean beetle prepupal weights significantly higher for stung but unsuccessfully parasitised larva (62.09 mg) than those successfully parasitised (52.94 mg). The chance of an emerged wasp larva spinning a pupal cocoon was found to increase by 5% with every 1-mg increase in its beetle prepupal weight. Heavier beetle prepupae produced bigger parasitoids. Field-collected adult *E. daenerys* had larger (12%) head capsule widths (mean of 1.42 mm) than those reared in the laboratory (mean of 1.27 mm), suggesting that improving host nutrition and laboratory rearing conditions for increasing host size may help optimise *E. daenerys* rearing success.

## KEYWORDS

biocontrol, flight, host size, mating, parasitoid, weather

## INTRODUCTION

Understanding the biology of a parasitoid and predicting its host range in the field is paramount to gaining approvals and to achieving a successful classical biological control program. Recent studies have promoted post-release monitoring of effectiveness to validate predictions of biological control introductions and ensure that bio-control continues to be socially accepted (Mills & Kean 2010; Schröder et al. 2020). Design of an effective post-release monitoring program requires knowing when flight activity of the parasitoid is likely to occur and, during this period, what environmental factors such as temperature, humidity and wind speed will influence adult wasp flight (Rousse et al. 2009). Although insects are known to have circadian patterns of activity, almost exclusively this research has been laboratory based with ecological implications extrapolated from such studies (Bertossa et al. 2013; Chen et al. 2020; Sanders & Lucuik 1975). Determining the interactions of abiotic factors with flight activity directly in the field is more pertinent than the laboratory as a method to increase the success of rearing conditions, field release methods and subsequent field monitoring programs (Coelho et al. 2016). Whipple et al. (2013), for example, found significant differences in sweep net catches of grasshoppers during different times of the day and by doing so demonstrated the need for standardised sampling times for rangeland grasshopper management. Furthermore, knowing the flight activity of parasitoids has important implications for integrating biological control with other pest management methods, thereby increasing the potential for success (Chen et al. 2020).

Many hymenopteran parasitoid mating systems are insufficiently studied in the field and are generally poorly understood, with their classification typically simplistic compared to other taxa (Boulton et al. 2015). They have a haplodiploid sex determination system whereby unmated females will only produce male offspring resulting in rapid laboratory colony failure (Heimpel & de Boer 2008; Schneider & Viñuela 2007). One relevant aspect of hymenopteran mating systems is the strategies they may use for mate location. They have been found to use one or more methods including pheromones, male lek formation, remaining at or near their emergence site, or actively searching for female emergence, oviposition or feeding sites (Ayasse et al. 2001; Boulton et al. 2015). Sex-specific circadian differences in adult eclosion, adult activity, reproductive cycles and mating receptivity may all provide evidence towards the mating system used (Herberstein et al. 2017). The parasitoid that we are studying in the field in its area of origin (Tasmania) is *Eadya daenerys* Ridenbaugh (Hymenoptera: Braconidae: Euphorinae). It will mate in the laboratory (M. Davy et al., unpublished data), and there is a record in Rice (2005a) of multiple males flying downwind of females sitting on

foliage in the field, which is insufficient to substantiate the mating system.

Mating is just one of the aspects of parasitoid ecology that is important in a prospective biological control agent. It is also important to understand how the relationship between host size and subsequent parasitoid survival and fitness aids in the selection of optimal host sizes for laboratory culturing and therefore optimal laboratory rearing success. There are two feeding strategies utilised by koinobiont parasitoids: tissue feeding or haemolymph feeding, with wasp larvae pupating either within or externally to their host's body, respectively (Harvey & Malcicka 2016). Many koinobiont parasitoids including *Campoletis sonorensis* Cameron (Hymenoptera: Ichneumonidae) utilise tissue-feeding strategies, which involve the larvae consuming most of the host tissue before they pupate (Harvey & Malcicka 2016). However, large hosts often provide far more resources than needed by tissue-feeding larvae, meaning they are often forced to overeat (Harvey & Strand 2002). This overfeeding is assumed to occur to enable successful pupation, which can only be achieved in the drier environments provided when most of the host is consumed (Harvey & Malcicka 2016). Haemolymph feeding, however, involves the parasitoid larvae feeding on the haemolymph and fat bodies of its host. Once maturity is reached, they use their mandibles to cut a hole in the host cuticle, to emerge through and pupate outside of their host (Malcicka & Harvey 2015). This enables haemolymph feeders to exit their host when needed, thereby benefiting parasitoid growth, development and survival (Harvey & Malcicka 2016).

These two developmental strategies depend on maximising body size, development time or a trade-off between the two (Beckage et al. 2012). Parasitoids that prioritise maximising their body size in a smaller host will delay their development by allowing its host to grow to an adequate size, thus enabling increased parasitoid development (Pennacchio & Strand 2006). Therefore, the development time from oviposition to pupa will increase, and once the adequate host size is reached, the larval growth rate will increase due to greater resource availability (Beckage et al. 2012). Prior research on *E. daenerys* (when it was believed to be *Eadya paropsidis* [Rice 2005a]) indicates that this parasitoid is a haemolymph feeder and that it can develop fully after oviposition in any of the four larval instars, but in different durations (Rice 2005b), suggesting that it prioritises maximising body size. Research on development time and size was previously undertaken on the primary beetle host in Tasmania, whereas once released in New Zealand, *E. daenerys* will be utilising a much larger host beetle, the target of the biological control. We will examine the relationship between growth rate and attained body sizes in relation to host size using both host species.

*E. daenerys* is a univoltine larval koinobiont endoparasitoid approved in 2019 for release as a biological control

agent against the invasive leaf beetle *Paropsis charybdis* Stål in New Zealand (Withers et al. 2021). *E. daenerys* is one of the several Australian species of *Eadya* that are larval endoparasitoids of eucalypt-feeding chrysomelids (Peixoto et al. 2018). In Tasmania, *E. daenerys* is most commonly reared from *Paropsisterna agricola* Chapuis and to a lesser extent *P. charybdis* (Peixoto et al. 2018). It is active for a very short time window after emerging from the soil where it has spent around 10 months as a pupa having exited its host's prepupal stage (Pugh et al. 2020). Other minor hosts of *E. daenerys* include *Paropsisterna bimaculata* Olivier and *Paropsisterna nobilitata* Erichson (Peixoto et al. 2018). Because no other recorded hosts are present in New Zealand, *E. daenerys* will utilise the larger *P. charybdis* as its primary host (Pugh et al. 2020). In New Zealand, *P. charybdis* undergoes two generations each year (Withers & Peters 2017), but it is essential for this biological control program to be effective that the parasitoid flight season overlaps with the first generation of the target as has been predicted (Pugh et al. 2020).

Finally, the hosts of *E. daenerys* vary greatly in adult body size, and this may impact upon the developmental success of the wasp (Withers et al. 2020). In New Zealand, the target beetle *P. charybdis* is 40% larger than *Pst. agricola*, so we aim to explore the relationship between host size and parasitoid development. The host size relationship for *E. daenerys* development is important as it has already been used to interpret the results of non-target host specificity testing conducted on non-target species of chrysomelid beetles of varying sizes in New Zealand (Withers et al. 2020, 2021).

The aims of this research were to examine daily (circadian) patterns of male and female *E. daenerys* flight activity in the field in relation to weather conditions and adult host beetle activity to help inform release locations, post-release field monitoring and the likely mating system being used by *E. daenerys*. In the laboratory, our aim was to improve rearing success by investigating the relationship between parasitism success, the host beetle's prepupal weight from which the wasp larva emerges, and the relationship between emergent wasp size and wasp fecundity.

## MATERIALS AND METHODS

### Male and female flight activity of *E. daenerys* in the field

The main field study site used from 2014 to 2017 was within a 426-ha forestry plantation located at Runnymede, in southern Tasmania (42.6479° S, 147.5549° E, 233 m), where high populations of both the parasitoid and one of its significant hosts *Pst. agricola* were observed (Peixoto et al. 2018). The plantation contained a 2007 planting of *Eucalyptus nitens*, which were approximately 4–7 m in height over the collection

window with an understory of mixed-grass species. In 2014, during initial exploration, two other *Eu. nitens* plantations were used for netting adult *E. daenerys* (Ellendale in southern Tasmania [42.6167° S, 146.7167° E] and Moina in northern Tasmania [41.5408° S, 146.0772° E]). Further collections were made in 2019 at a southern Tasmanian *Eu. nitens* plantation in Buckland (42.6231° S, 147.7249° E) and in 2020 in addition to Buckland at five more *Eu. nitens* plantation sites all in northern Tasmania: Kimberley (41.4126° S, 146.4360° E), Dunns (41.2931° S, 147.3538° E), Coppermine (41.3008° S, 146.7586° E), Bessy (41.7132° S, 146.5945° E) and Dunning's (41.7105° S, 146.5981° E). Collection times (AEST time) ranged from 5:30 AM to 8:00 PM with 123 h of netting flying wasps moving between trees and a total of 57 independent trips undertaken (maximum collecting duration of 6 h). Successful collections were made between 17 November (Day 1) and 17 December (Day 32) across all seasons, and a total of 872 *E. daenerys* netted. Between 1 and 3 experienced netters using 380-mm-diameter hoop extendible-handle nets of up to 2 m in length were used per collection interval. Each female *E. daenerys* was transferred from the net into a glass vial (2 × 10 cm) with a ventilated lid, which was then put into an insulated cooler that contained frozen ice bricks to keep them cool before returning to the laboratory. Not all male *E. daenerys* were returned to the laboratory with many released from collecting vials at the end of that day's collecting. Parasitism of beetle larvae was not assessed but has been previously assessed at Moina, Runnymede and Ellendale in previous seasons (Peixoto et al. 2018).

The collection date, collection time, average wind speed (range 0–5.0 m/s), maximum wind speed (range 0.5–11.40 m/s), temperature (range 9.8–24.0°C), humidity (range 24.8–97.7), light intensity (2–507 lx in shade), number of collectors (between 1 and 3) and sex of wasp were recorded throughout the collection periods. These recordings were undertaken to determine the sex ratio of flying wasps throughout the season and the ideal range of environmental conditions that may underlie adult flight activity. The light intensity was recorded using a light metre (TPS MC-88) and the wind speed using an anemometer (Testo 425). One i-Button™ temperature and humidity data logger (DS1923) was placed within a Stevenson screen suspended from a tree limb 1 m above soil level of each collection period to monitor air temperatures and humidity. Weather data from the closest Australian Bureau of Meteorology weather station (42.6867° S, 147.4258° E, 45 m; the closest at 12.7 km from Runnymede) were sourced to fill in gaps in recorded data from Runnymede.

Malaise traps were deployed in the 2015 season to get an independent measure of *E. daenerys* flight activity and over a more continuous time window compared to the net catches. This collection also provided wasps to enable comparison of the distribution of field wasp size and fecundity in relation to laboratory measures of wasp

size detailed in the next section. Four malaise traps were set up on the 17 November 2015 to the 17 February 2016 at different locations throughout the Runnymede plantation. The traps were placed approximately 100 m apart. All traps were erected directly under or close to eucalypt trees, adjacent to clearings. The malaise collection bottles were filled with 250 mL of 70% ethanol and cleared weekly. The contents of the malaise traps from each week's collections were taken back to the laboratory, and the total number of male and female *E. daenerys* and the confirmed hosts *Pst. agricola*, *P. charybdis* and *Pst. bimaculata* adult beetles was recorded. Wasps were stored in 70% ethanol in a refrigerator before measuring their head capsule width and counting the number of mature eggs in the female *E. daenerys*.

### The relationship between parasitism success and the beetle prepupal weight of its host

To investigate beetle prepupal weights on parasitism success, one field-collected adult female *E. daenerys* was placed in a 25 × 20 × 20-cm sleeve cage with a eucalypt leaf (*Eu. nitens*) located on the bottom of the cage upon which approximately 10 second-instar beetle larvae were settled. Larval instars were determined by both larval size and presence of shed exoskeletons. Second-instar larvae are the preferred host instar for laboratory rearing of *E. daenerys*, reflecting both a field preference for early instars and better parasitoid rearing success than other instars (Rice 2005b; Rice & Allen 2009). Larvae were obtained from eggs laid by field-collected beetles, which were then reared on cut foliage at 20 ± 1°C and 16:8 L:D until reaching the second instar.

Female *E. daenerys* and beetle larvae of *Pst. agricola* and *P. charybdis* were observed continuously, and as soon as each larva had been oviposited into, it was removed with a camel-hair brush and subsequently placed into an unventilated, paper towel-lined plastic takeaway container (1.2 × 17.5 × 4.5 cm) along with young eucalypt leaves free from other insects. Oviposition was designated when the wasp inserted its ovipositor into a larva for at least 1 s, as was used in Withers et al. (2020) where 94% of *P. charybdis* oviposited into were later found to be parasitised. Immediately following oviposition, each stung larva was replaced with a new unparasitised larva. Larvae were continued to be replaced until the female *E. daenerys* stopped ovipositing for a period of 10 min. A total of 41 field-collected female *E. daenerys* were used to set up the experiment.

Once stung, all larvae were reared at 20 ± 1°C and ~70% RH. The eucalypt leaves were replaced every second day, and the paper towel was replaced ad libitum until all larvae had either reached prepupae or died. Once beetle larvae reached their prepupal stage, the date was recorded, and they were weighed to the nearest 0.1 of a milligram (Mettler AE260 delta range). Each weighed larva

was placed into a 2 cm × 1.5-cm pocket made from 'trace and toile' interfacing fabric, which had been folded in half and stapled around three sides to facilitate successful wasp pupation. If the prepupa developed into a beetle pupa, it was again weighed, pupation date recorded and placed into a plastic vial until adult emergence. If an *E. daenerys* larva emerged and spun a silk pupal cocoon, the newly spun cocoon was weighed within 48 h of spinning and put in a plastic vial (5 × 1.8 cm) with a loose-fitting screw lid. The plastic vials containing *E. daenerys* pupal cocoons were then returned to the same temperature cabinet with the same photoperiod and temperature for larval rearing.

### Female wasp size and fecundity

To enable comparison of *E. daenerys* sizes between different host species, host sizes and field and laboratory rearing conditions, the head capsule widths of adult wasps that were collected during the 2015 season were measured. These specimens included those wasps collected in the field with nets and in malaise traps and those that had emerged or were dissected out of pupal cocoons reared in the laboratory from *Pst. agricola* and *P. charybdis*. A digital image of their head capsule was taken through a stereo microscope at 4× magnification using a Canon EOS 600D camera and head capsule widths measured to the nearest 0.01 mm using ImageJ software (Rasband 1997).

To determine the egg load of an individual female and if this changes with size or differed for those female *E. daenerys* collected early in the day compared to the rest of the day, the number of mature eggs (determined by the larger size, clear ovoid shape and lower position in oviduct) that a female had (either caught by netting or caught in a malaise trap) was counted with the help of a grid graticule. This was achieved by suspending the female in 70% ethanol in a petri dish (10 × 1.5 cm) and dissecting her abdomen under a stereo microscope at 40× magnification.

### Statistical analysis

All data were statistically analysed using IBM SPSS statistical package 22 or R open-source statistics Version 2.15. Flying *E. daenerys* caught by net were partitioned into 491 15-min intervals starting on the hour with the number of male and female wasps caught per quarter hour divided by the number of collectors recorded along with the corresponding average air temperature, humidity, average wind speed, maximum wind gust speed and light intensity for each quarter hour. The 32-day flight season was divided into two halves of 16 days each (commencing 17 November) and, within each half of the season, catch rates per quarter hour allocated to one of four daily



time periods (5:30 AM to 9:00 AM, 9:00 AM to 1:00 PM, 1:00 PM to 5:00 PM and 5:00 PM to 8:00 PM all AEST). The flight season was split into two halves to enable testing for possible seasonal shifts due to protandry, which is common in parasitoid wasps (Boulton et al. 2015), whereas we had sufficient data to split the day into four time periods, thereby partitioning the dawn and dusk shifts in light intensity from the daytime. The median proportion of each catch that was female (i.e., the sex ratio) per daily time period in each half of the season was calculated using the overall sex ratios of each trip, where at least four *E. daenerys* were caught in that daily time period. The median catch rates per quarter hour of each sex for each daily time period in each half of the season were calculated using the overall averages of each trip in that daily time period. Medians were used as the data were not normally distributed with many zero catch time periods evident. Differences in both sex ratio and catch rates between daily time periods and season halves were tested for using Kruskal–Wallis tests. As *E. daenerys* had distinct flight patterns within a day between daily time periods, the parameters that varied predictably during the day (i.e., either higher during 9:00 AM–5:00 PM [air temperature and light intensity] or lower [humidity]) were not used in subsequent analysis but reported for the ranges *E. daenerys* were found flying in. A stepwise linear regression was used to test whether average wind speed and maximum wind gust speed were significant predictors for the rate of male or female *E. daenerys* flight activity per quarter hour across any time of day and binary logistic regression used to determine the relationship of the same parameters to the probability of male and female flight per quarter hour across any time of day. Recursive partitioning decision tree analysis using the ‘party’ package and the ‘ctree’ function was also undertaken using the same predictors to determine a cutoff wind speed for chance of flying.

The relationship between host beetle prepupal weight of *Pst. agricola* and wasp pupal success was analysed using post-fitting for linear models (PLM), least square means and logistic regression. The host beetle prepupal weight and fecundity relationships with adult *E. daenerys* size were analysed with a Pearson coefficient and Mann–Whitney tests. Comparisons of head capsule widths between the sexes of *Pst. agricola* and *P. charybdis*-reared *E. daenerys* were analysed using ANCOVA with beetle prepupal weight as a covariate and sex as a factor.

## RESULTS

### Male and female flight activity of *E. daenerys* in the field

Net catches showed marked differences in the daily and seasonal patterns of flight between male and female *E. daenerys* (Table 1). The sex ratio of flying *E. daenerys* is

male biased (31% to 48% female) in the first half of the season across the first three daily time periods but significantly shifts to 70% females during the evening (5:00 PM–8:00 PM) ( $\chi^2 = 12.26$ ,  $p = 0.007$ ). In the second half of the season, both early morning and evening flights have significantly higher female sex ratios, whereas from 9:00 AM to 5:00 PM, it is male bias (11%–22% female) ( $\chi^2 = 21.07$ ,  $p = 0.0001$ ). In the evening, the median sex ratio significantly shifts from 70% to 100% female from the first half to the last half of the season, respectively ( $\chi^2 = 8.82$ ,  $p = 0.003$ ).

Catch rates of *E. daenerys* were never high. Across all daily time periods, no wasps were collected on 17.6% ( $n = 68$ ) of time periods. An average catch rate exceeding one wasp per quarter hour per time period occurred on only 10.3% (maximum 5.8) for males and on only 17.6% of occasions (maximum 4.3) for females. The median catch rates of *E. daenerys* similarly showed marked daily and seasonal differences between male and female wasps (Table 2). In the first half of the season, the male catch rate significantly declined in the last two periods of the day ( $\chi^2 = 7.82$ ,  $p = 0.05$ ), whereas in the second half of the season, there was a significant decline in male catch rate during the early morning and especially during the evening ( $\chi^2 = 13.33$ ,  $p = 0.004$ ). The catch rates of females were higher in the first half of the season during the early morning and evening, although not significantly ( $\chi^2 = 3.95$ ,  $p = 0.27$ ), whereas in the second half of the season, female catch rates were significantly higher in the evening compared to the rest of the day ( $\chi^2 = 14.51$ ,  $p = 0.002$ ). In both the early and late seasons, female catch rates were higher than those of males in the evening (Wilcoxon signed-rank tests:  $Z = 2.37$ ,  $p = 0.018$  and  $Z = 2.42$ ,  $p = 0.016$ , respectively) with male evening catch rates also significantly higher in the first than those in the second half of the season ( $\chi^2 = 4.30$ ,  $p = 0.038$ ). There was a significantly higher catch rate (1.6 per quarter hour) of wasps in the early morning in the first half of the season compared to the second half (0.35 per quarter hour) of the season ( $\chi^2 = 4.36$ ,  $p = 0.037$ ).

*E. daenerys* were found flying across a wide range of air temperatures (10.7–23.8°C), humidity (24.8%–97.7%) and light levels (2–400 lx in shade), which all have predictable daily patterns, as well as a wide range of average wind speeds (0.03–2.87 m/s) and maximum wind gust speeds (0.50–8.19 m/s: calm–fresh breeze). Both average wind speed ( $\beta = -3.12$ ,  $p < 0.0001$ ) and maximum wind gust speed ( $\beta = 0.068$ ,  $p < 0.001$ ) were significant predictors of the rate of male flight activity across any time of day using stepwise linear regression ( $F_{2, 404} = 30.05$ ,  $p < 0.0001$ ). Logistic binary regression identified average wind speed as a significant predictor ( $\chi^2 = 41.72$ ,  $p < 0.0001$ , 74% correctly classified) indicating that for every 1-m/s increase in average wind speed, the odds of catching a male dropped 0.23-fold. Recursive partitioning similarly showed average wind speed to be the strongest predictor of the chance of male flight ( $p = 0.0001$ ). When

**TABLE 1** Median proportion of *E. daenerys* catch that is female over collecting trips where at least four wasps were caught in the time period pooled over six flight seasons.

Days in season	5:30 AM–9:00 AM	9:00 AM–1:00 PM	1:00 PM–5:00 PM	5:00 PM–8:00 PM	
1–16	0.31 <sub>a</sub>	0.48 <sub>a</sub>	0.38 <sub>a</sub>	0.70 <sub>b</sub>	$p = 0.007$
17–32	0.55 <sub>a</sub>	0.22 <sub>b</sub>	0.1 <sub>b</sub>	1.00 <sub>a</sub>	$p = 0.0001$
	<i>ns</i>	<i>ns</i>	<i>ns</i>	$p = 0.003$	

Note: Number of independent collections per time period: Days 1–16: 3, 6, 2 and 7; Days 17–32: 5, 9, 3 and 12, respectively. Total number of wasps caught: 856. Where  $p < 0.05$ , different subscript letters denote significant differences between medians. *ns* denotes not significant.

**TABLE 2** Median catch rate per 15 min per person for male and female *E. daenerys* over collecting trips where at least one wasp of either sex was caught in the time period pooled over six flight seasons.

Days in season	5:30 AM–9:00 AM	9:00 AM–1:00 PM	1:00 PM–5:00 PM	5:00 PM–8:00 PM	
♀ 1–16	0.73	0.25	0.13	0.59 <sub>a</sub>	$p = 0.27$
♂ 1–16	0.88 <sub>a</sub>	0.59 <sub>ab</sub>	0.00 <sub>b</sub>	0.16 <sub>b</sub>	$p = 0.05$
♀ vs. ♂	<i>ns</i>	<i>ns</i>	<i>ns</i>	$p = 0.018$	
♀ 17–32	0.15 <sub>a</sub>	0.19 <sub>a</sub>	0.37 <sub>ab</sub>	1.20 <sub>b</sub>	$p = 0.002$
♂ 17–32	0.07 <sub>ac</sub>	0.58 <sub>b</sub>	0.87 <sub>ab</sub>	0.00 <sub>c</sub>	$p = 0.004$
♀ vs. ♂	<i>ns</i>	<i>ns</i>	<i>ns</i>	$p = 0.016$	

Note: Number of independent collections per time period: Days 1–16: 2, 6, 5 and 8; Days 17–32: 8, 9, 4 and 13, respectively. Total number of wasps caught: 872. Where  $p < 0.05$  different subscript letters denote significant differences between medians. *ns* denotes not significant.

average wind speeds were less than 0.27 m/s, 60% ( $n = 85$ ) of the classified catch times had flying males, and when greater than 0.27 m/s, only 18% ( $n = 335$ ) of the classified catch times had flying males.

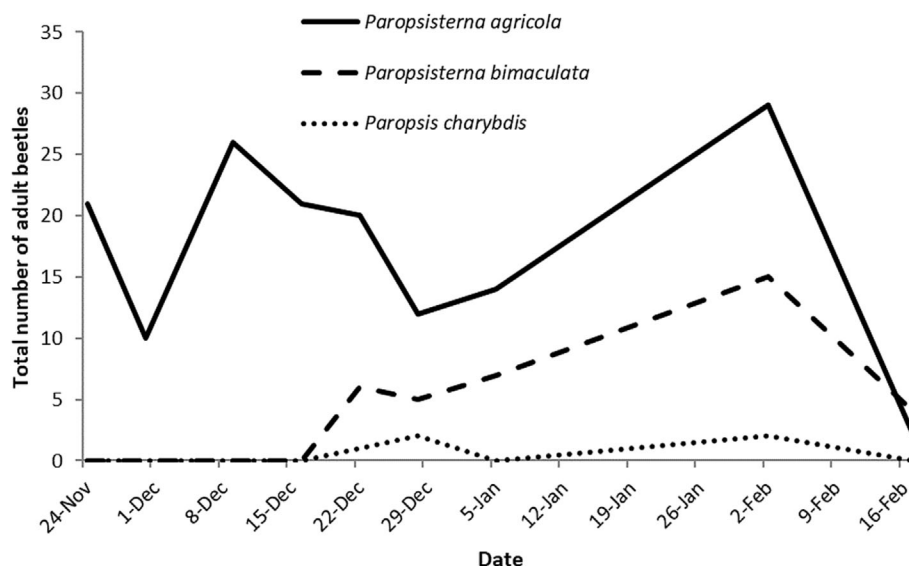
Maximum wind gust speed ( $\beta = -0.051$ ,  $p = 0.0004$ ) was a significant predictor of the rate of *E. daenerys* female flight activity across any time of day ( $F_{2, 404} = 12.66$ ,  $p = 0.0004$ ). Recursive partitioning showed both average wind speed ( $p = 0.003$ ) and maximum wind gusts ( $p = 0.021$ ) to be significant predictors of the chance of female flight. When average wind speeds were greater than 0.73 m/s, just 24% ( $n = 226$ ) of females were found flying. Where average wind speed was less than 0.73 m/s, the maximum wind gust speed was a further predictor of flight. Only on 17% ( $n = 38$ ) of catch times were females flying when average wind speed exceeded 2.67 m/s (light breeze), whereas on 57% ( $n = 156$ ) of catch times were females flying when maximum wind gusts were below this speed.

Malaise trapping in the 2015 season showed that for both sexes, the population of *E. daenerys* declined rapidly over their flight season with males declining much more rapidly than females. In the week of 17–24 November, we caught 27 females and 27 males. Over the next 3 weeks, we caught 12, 7 and 0 females and 4, 1 and 0 males, respectively. Despite traps being set each week until 17 February, no further wasps were caught. Netting undertaken before 17 November, the day malaise traps were set, found no flying *E. daenerys*, suggesting that numbers were too low to net prior to this date and that the flight season start was on or shortly before 17 November in that year. The observed adult flight season for all three known

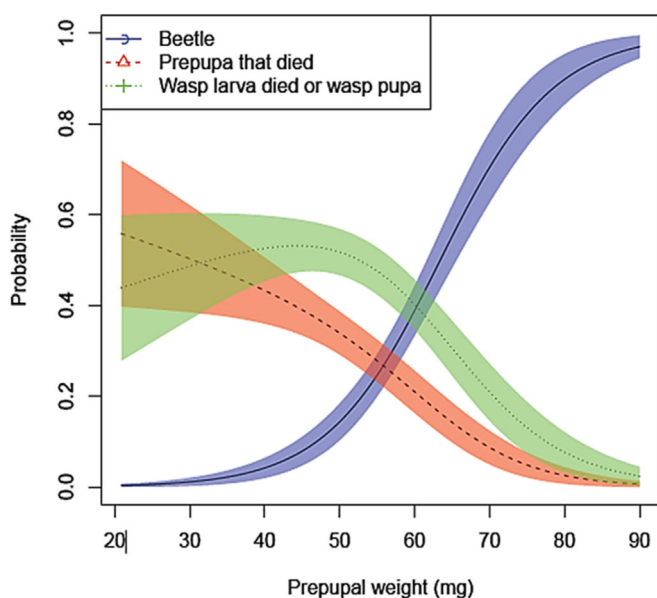
host beetle species found at Runnymede extended much longer than that of *E. daenerys*. The most abundant beetle species *Pst. agricola* had the longest season of at least 92 days, with adult beetles still being caught until the week commencing 10 February 2016 when the malaise traps were dismantled (Figure 1). Adult *Pst. bimaculata* and *P. charybdis* flight seasons started later, beginning on the week commencing 15 December 2015, and were still being observed when the malaise traps were dismantled. The total counts for each of the three leaf beetle species peaked on 2 February 2016 and rapidly decreased thereafter, corresponding with the emergence of the  $F_1$  generation of adults, which had been larvae in the preceding months.

## Host weight and parasitism success

In *P. charybdis* larvae, the mean number of days from female *E. daenerys* stinging to beetle prepupal development was significantly longer for parasitised than non-parasitised beetle larvae:  $15.6 \pm 0.7$  (SE) days for non-parasitised larvae and  $18.4 \pm 0.1$  days for parasitised larvae ( $t_{438, 452} = -10.79$ ;  $p < 0.001$ ). Whereas, in *Pst. agricola* larvae, the range of days post-stinging to developing into a beetle prepupa was  $13.4 \pm 0.3$  days and was not significantly different between larvae that were successfully parasitised and those that were not ( $t_{377, 582} = 1.05$ ;  $p = 0.30$ ). The beetle prepupal weight of *Pst. agricola*, which ranged from 20.9 to 88.9 mg (mean  $55.9 \pm 0.6$  mg [SE],  $n = 404$ ), was a significant predictor of the outcome of parasitism ( $\chi^2 = 153.72$ ,  $df = 3$ ,



**FIGURE 1** Weekly adult total malaise trap counts for three paropsine leaf beetle hosts of *E. daenerys* at Runnymede for the 2015/2016 season.



**FIGURE 2** Fitted logistic curves with 95% confidence intervals for the effect of *Paropsisterna agricola* beetle prepupal weight (mg) for three post-beetle prepupal outcomes (dead beetle prepupa, beetle or *E. daenerys* wasp).

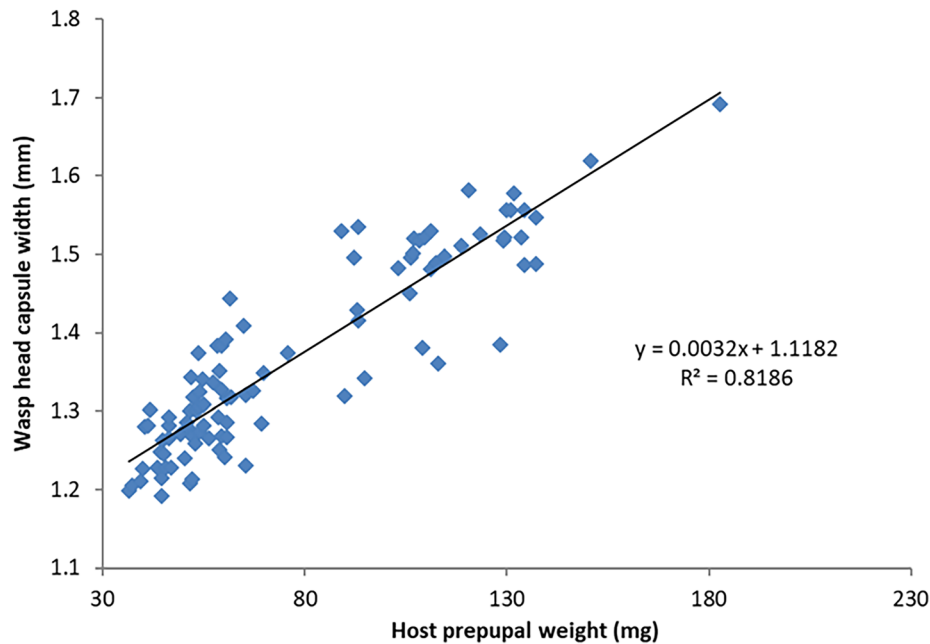
$p < 0.001$ : Figure 2). The prepupal weights of beetles that were successfully parasitised or died as a beetle prepupa were significantly lower than those that were not successfully parasitised and became a beetle pupa. *E. daenerys* wasp larvae that successfully spun a pupal cocoon after emerging from the beetle prepupa had significantly higher host beetle prepupal weights than those that died as a wasp larva and failed to spin a wasp pupal cocoon. For every 1-mg increase in host beetle prepupal weight, the probability of an emerged *E. daenerys* larva successfully spinning a wasp cocoon

increased by 5% ( $\chi^2 = 99.18$ ,  $df = 2$ ,  $p < 0.001$ ). The logistic curves plotting the probability of the differing outcomes show that once beetle prepupae are beyond 60 mg, they have a high probability of being unparasitised, whereas beetle prepupae weighing up to 55 mg have about a 50% probability of a wasp larva emerging from them (Figure 2). The probability of a beetle prepupa dying before either *E. daenerys* larval emergence or becoming a beetle pupa is the highest among the smaller beetle prepupa and rapidly declines with increasing beetle prepupal weight.

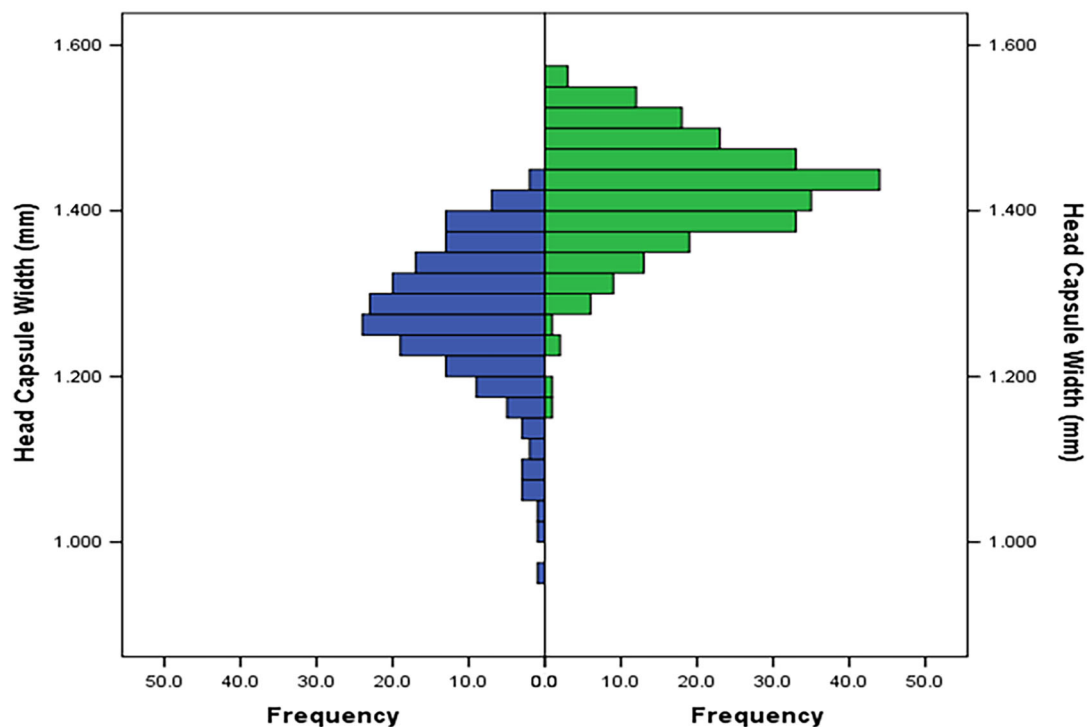
### *E. daenerys* size and fecundity

There was a significant ( $p < 0.001$ ,  $y = 0.0032x + 1.1182$ ,  $R^2 = 0.81$ ) positive relationship between host prepupal weight and the head capsule width of the adult *E. daenerys* that emerged from it, with larger host weights resulting in larger adult wasps (Figure 3). However, the head capsule widths of *E. daenerys* that were collected from the field were significantly larger by 12% ( $1.42 \pm 0.005$ ,  $n = 253$ ) than those reared from *Pst. agricola* on cut foliage in the laboratory ( $1.27 \pm 0.006$ ,  $n = 179$ ) ( $Z_{1, 430} = 395.01$ ,  $p < 0.001$ ) (Figure 4). *E. daenerys* reared in the laboratory out of *P. charybdis* reared on cut foliage were significantly larger ( $1.48 \pm 0.01$  mm,  $n = 61$ ) than those reared out of *Pst. agricola* in the field or in the laboratory ( $Z_{2, 490} = 240.9$ ,  $p < 0.001$ ). There was a marginal significant difference between the head capsule widths of male and female *E. daenerys* reared in the laboratory from *Pst. agricola* ( $F = 5.55$ ,  $df = 1.60$ ,  $p = 0.02$ , male =  $1.27 \pm 0.01$  mm, female =  $1.30 \pm 0.01$  mm), but this relationship was not evident in the field-collected individuals.

In field-collected females, there was no significant relationship between adult *E. daenerys* head capsule



**FIGURE 3** Host beetle prepupal weight (mg) (using both *Paropsisterna agricola* <80 mg and *Paropsis charybdis* >80 mg) and the head capsule width (mm) of laboratory-reared *Eadya daenerys* across both host species ( $n = 96$ ).



**FIGURE 4** Head capsule width (mm) of adult *Eadya daenerys* (left) reared in the laboratory on *Paropsisterna agricola* ( $n = 179$ ) or (right) collected in the field ( $n = 253$ ).

width and the number of mature eggs in a female, with the number of mature eggs ranging from 0 to 618 eggs ( $y = -69.828x + 413.38$ ,  $R^2 = 0.001$ ,  $p > 0.05$ ). However, there was a significant difference between the number of mature eggs and the time period of day that the female was caught ( $\chi^2 = 15.02$ ,  $df = 2$ ,  $p = 0.02$ ). Females

caught in the early morning averaged  $134.2 \pm 27.6$  ( $n = 15$ ) mature eggs, which was significantly fewer than those caught during the day ( $331.3 \pm 32.7$ ,  $n = 28$ ) and at dusk ( $248.2 \pm 37.0$ ,  $n = 13$ ), suggesting that many female wasps caught in the early morning were newly emerged. Indeed, the only field mating ever seen was



around 9:00 AM in the first half of the season on a leaf surface.

## DISCUSSION

Classical biological control projects benefit from ecological information on the parasitoid from its country of origin. As is often the case in biological control, little was known of this parasitoid *E. daenerys* prior to the attempted biological control of *P. charybdis*. As had occurred during the collection of *Anaphes* spp. in Australia and its application as a biological control agent (Schröder et al. 2020), *Eadya* spp. was found to be a confused species complex. Before biological control could proceed, research included natural enemy development (Rice 2005b), phenology (Rice & Allen 2009), species identity and descriptions (Ridenbaugh et al. 2018) and ecological host range (Peixoto et al. 2018). The current research investigated circadian and seasonal flight activity in the field and quantified laboratory rearing success. Both aspects will be important for implementing an effective post-release monitoring program and to inform mass-rearing methods for releases. This will be further discussed below.

*E. daenerys* were netted in Tasmania flying over a 32-day window from mid-November to mid-December during six differing field seasons. The observed adult flight season for all three known host beetle species extended much longer than that of *E. daenerys*. Indeed, *E. daenerys* is synchronised with the first larval generation of its most abundant host *Pst. agricola* but then enters an obligate pupal diapause in the leaf litter until the flight season for the following year (Rice 2005b). That males were more active in the first half of the season is most likely due to males having emerged earlier than females. This occurs in the laboratory population of *E. daenerys*, with first male emergence and peak male emergence consistently preceding female emergence by between 4 and 7 days (M. Davy, unpublished data). Males emerging significantly earlier than females indicates protandry in *E. daenerys*, which is a strategy hypothesised to increase male mating success and reduce the risk of female pre-reproductive death (Quicke 1991; Waage & Ming 1984).

There was a declining level and catch rate of males as the season progressed, which indicates a shorter male lifespan. Similar to other studies on hymenopteran parasitoids (He et al. 2004), this pattern may be adaptive to ensure that seasonal peaks in mating and oviposition do not fully overlap in *E. daenerys*. This seasonal activity could have a role in preventing males from harassing females once their oviposition activity starts. Male harassment during oviposition can reduce female fitness in insects (e.g., Li et al. 2014) including in parasitoids (Boulton & Shuker 2015).

Wasps were found flying across a wide range of air temperatures, humidity and light levels with flight occurring successfully at even the very lowest light levels (2 lx) present at sunrise and sunset. We did not collect during periods of rainfall, and the influence of barometric pressure, which has been recorded to impact flight activity of other parasitoid wasps, is yet to be investigated (Rousse et al. 2009). *E. daenerys* were observed to be weak fliers with even light winds significantly reducing recorded flights when average wind speed exceeded 0.27 m/s for males and 0.73 m/s for females.

The early morning in the first half of the season saw flight skewed towards a male bias sex ratio with the catch rates of males the highest per quarter hour of all time periods. Scramble competition for mates (Nahrung & Allen 2004) appears to describe the mating system of *E. daenerys* with males actively searching and competing for virgin females who signal by pheromones receptivity to mating. Male traits associated with scramble competition in terrestrial arthropods typically include high mobility, the ability to detect and locate females over long distances, and protandry, which all increase the frequency of encounter rates with widely dispersed females (Herberstein et al. 2017). No exaggerated male traits are obvious in *E. daenerys*, but any trait that promotes high flight endurance in males, resulting in longer searching times and higher success in locating females, would increase reproductive success. Female *E. daenerys* flying in the early morning had low mature egg loads compared to daytime or evening flying females, indicating that they may be younger and most probably newly emerged. The majority of koinobiont parasitoids such as *E. daenerys* are predicted to be carrying mature eggs when they emerge from pupation though in lower numbers at first, with the rest maturing 2 to 3 days post-emergence (Jervis et al. 2001; Khatri et al. 2009). When comparing the first to second half of the season, the early morning had the most significant drop off in wasps of both sexes flying with a 4.5-fold decrease in the second half of the season.

Daytime flying of *E. daenerys* was not the peak time for female flight, with sex ratios being especially male biased in the latter half of the season. It may be that males continued to search for females especially in the morning (9:00 AM–1:00 PM) but, as the season progressed, also into the afternoon. However, the evening period showed a significant increase in female flight activity so that by the second half of the season, it was almost exclusively female, with over 3× more females flying in the evening than at any other period of the day. High female activity in the evening suggests it may be a peak oviposition period for female *E. daenerys* with timing possibly linked to female avoidance of males and any ensuing sexual conflict (Lee et al. 2021). The netting results are also invaluable for informing the timing of any post-release monitoring program to ascertain establishment of *E. daenerys* in the field in New Zealand.

When attempting to establish a new species of biological control agent, destructive sampling catches using malaise traps with ethanol collection bottles may vary according to trap orientation, placement and height of surrounding vegetation but worse would also create unwanted mortality effects to the founder population (Matthews & Matthews 1971). Instead, this research shows that netting would be an effective method early in the season (mid-late November) to catch both males and females, if conducted at either dawn or dusk (Table 2). Adults could then be identified and re-released unharmed. If netting continued into mid-December, evening would be the most effective time to catch flying females, who could then also be re-released.

Mass rearing the parasitoid for release is a very important aspect to ensure success of the biological control project in New Zealand. With the obligate univoltine nature of *E. daenerys*, there is just one opportunity per year at laboratory rearing to ensure that a population of high-quality wasps is being bred for release. Beetle prepupal weight was found to be a predictor of wasp parasitism success. Mean beetle prepupal weights were significantly higher for unparasitised larva than those that resulted in a wasp larva that successfully spun a pupal cocoon, with the smallest prepupae having wasp larva emerge but failing to spin a pupal cocoon. The chance of a wasp spinning a pupal cocoon was found to increase by 5% with every 1-mg increase in beetle prepupal weight. Generally, koinobiont hymenopteran parasitoids cause their host to consume less food, slowing the host rate of development, which results in a lower final body weight than unparasitised hosts (Elzinga et al. 2003; Morales et al. 2007). This was evident for *E. daenerys* with parasitised *P. charybdis* larvae taking longer to reach prepupal stage than unparasitised larvae. The larger size of wasps emerging from the host *P. charybdis* also suggests that *E. daenerys* are haemolymph feeders as they continue to grow and consume the larger food resource by slowing the host's development to maximise their adult size and therefore fitness (Malcicka & Harvey 2015). It is hoped that the larger sized *E. daenerys* in New Zealand will equate to greater fitness, in terms of both flight ability and fecundity, and therefore greater reproductive success.

We now know that heavier beetles produce bigger wasps, but field-collected adult wasps were found in this study to have significantly larger (12%) head capsule widths than those reared in the laboratory. This suggests that the laboratory rearing protocols may not have been optimal for wasp rearing success. The main *E. daenerys* host species observed at Runnymede was *Pst. agricola*, and so it would be expected that their head capsule widths would not be significantly different from laboratory-reared wasps on the same host, but this was not the case. The cause for the significant difference may be due to the laboratory rearing conditions of constant 20°C, 16:8 L:D and 70% RH and the host being reared on cut foliage precipitating host and/or wasp stress. The

stress could reduce the prepupal weight, resulting in significantly smaller adult wasps. Laboratory rearing should attempt to avoid foliage quality being an issue by supplying fresh foliage in over-abundance. Adult leaf maturation should be carefully matched to the requirements of the *P. charybdis* larval instar and foliage changed every 48–72 h so that food is never limiting. Future experiments need to look at different rearing temperatures that fluctuate, as in the field, to see if this is a stressor. It could also be beneficial to attempt rearing on leaves in situ on coppiced trees to see if excising leaves can be alleviated. To date, leaves have had to be cut from the trees and stored prior to feeding as the adult foliage upon which *P. charybdis* larvae feed grows between 4 and 12 m high on *Eu. nitens*.

In other parasitoid species such as *Itopectis naranyae* Ashmead (Hymenoptera: Ichneumonidae), there is a positive linear correlation with female size and mature egg load (Liu & Ueno 2012). The larger the adult size, the more eggs she can carry, which increases her fitness (Visser 1994). Therefore, it would be reasonable to expect this to be so for *E. daenerys*. However, this relationship was not evident in field-caught females, which could have been due to the unknown age of the female field-collected wasps, the number of ovipositions they had already undertaken or low food availability. To obtain more reliable results, all dissected *E. daenerys* would need to be raised under laboratory conditions and their oviposition history and date of emergence recorded.

In summary, the field biology of *E. daenerys* is now better understood with net catch efficiencies known for different times of the day and during the season. We can now develop a reliable monitoring method to confirm whether the classical biocontrol release in New Zealand is successful. We now also know that initial release sites should target less windy locations and avoid very windy days, to maximise the chance of establishment. Sexes differ in their flight activity during the day, which aligns with a scramble competition mating system among males for females. Finally, as successful adult emergence is closely linked to host prepupal size and because laboratory-reared wasps are smaller than field-collected wasps, rearing protocols for beetle hosts need to be improved to maximise the size and fitness of wasps released for biocontrol.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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