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Predators, parasites and heterospecific aggregations in chrysomeline larvae

Running title: Heterospecific chrysomeline larval aggregations

Abstract

Gregariousness is a common feature in aposematic insect prey and offers the additional benefit of enhancing the effectiveness of their toxic defences. Aggregations of the aposematic larvae of two species of leaf beetles, *Paropsis atomaria* and *Paropsisterna variicollis*, occur together on the same Eucalyptus trees over spring and summer. Conventionally, the colouration of these larvae is thought to provide aposematic protection against vertebrate predators, but supporting evidence is limited. We determine if environmental preferences contribute to the heterospecific aggregations, and the potential costs for larvae of living in heterospecific aggregations. We surveyed natural aggregations of the larvae of both species in the field and recorded environmental variables of these aggregations. This revealed that heterospecific aggregations occurred more commonly at higher leaf temperatures, and in less visually conspicuous locations. *P. atomaria* larvae were twice as likely to be found in heterospecific aggregations than *Pa. variicollis*. Next, we manipulated larval aggregations in the field to investigate the survival and gregarious behaviour of larvae in heterospecific aggregations. The gregarious behaviour of both species of larvae did not differ between heterospecific and monospecific aggregations. Further, the survival of larvae did not significantly differ between heterospecific and monospecific aggregations. We suggest that the preference for *P. atomaria* larvae to aggregate with

Pa. variicollis at higher leaf temperature results in the observed heterospecific aggregations, with a potential benefit of lowerer parasitoidism rates for P. atomaria.

Keywords: Heterospecific aggregations, aposematic colouration, parasitoids, chrysomelines

Introduction

Gregariousness is widespread in insect larvae (Ghent 1960; Tsubaki 1981; Tsubaki & Shiotsu 1982; Wade & Breden 1986), and particularly benefits aposematic insects enhancing the effectiveness of their toxic defences (Cott 1940; Rettenmeyer 1970; Harvey et al. 1982; Sillen-Tullberg et al. 1982). Avian predators typically learn to avoid aposematic insects more rapidly and more strongly if they occur in aggregations (Gagliardo & Guilford 1993; Alatalo & Mappes 1996; Gamberale & Tullberg 1996). Accordingly, aposematic signalling has often evolved in gregarious prey species (Gagliardo & Guilford 1993; Alatalo & Mappes 1996; Alatalo & Mappes 2000), perhaps through a collective increase in the effectiveness of the aposematic signal (Gagliardo & Guilford 1993; Riipi et al. 2001). However, the reproducibility of colour patterns of individuals is expected to be high, if predators are to learn to avoid certain colour patterns, and deviations from these colour patterns are therefore not expected (Brower 1960; Brower et al. 1960; Rettenmeyer 1970; Sillen-Tullberg et al. 1982; Joron & Mallet 1998) but see Arias et al. (2016).

Aggregations with mixed phenotypes are unusual because individuals are not necessarily subject to the same risks of predation (Morse 1977). Heterospecific associations, reported in a range of taxa from phytoplankton to insects to dolphins (Terborgh 1990; Ohtsuka et al. 1995; Glos et al. 2007; Kiszka et al. 2011) see also review by Stensland et al. (2003), are thought to increase foraging efficiency and predator avoidance (Powell 1974; Barnard 1978; Kiszka et al. 2011; Bryer et al. 2013). However, these benefits and costs may not be similar for all individuals within heterospecific groups, and large deviations in colours and patterns among a few individuals within aggregations may lead to the so-called ‘oddity effect’ (Tosh et al. 2007; Rodgers et al. 2013). This occurs where a minority of individuals possess a distinctive (odd) phenotype that may reduce a predator’s sensory confusion brought on by a large group of uniform-looking prey (Landeau & Terborgh 1986). As a result, the probability of the predator targeting odd individuals increases, and they consequently suffer proportionately higher rates of predation (Landeau & Terborgh 1986; Krakauer 1995). This may lead to size-assortative grouping: fish, for example, are more likely to join shoals of individuals that match their size (Peuhkuri 1997; Peuhkuri et al. 1997; Peuhkuri 1999). However, different classes of individuals (e.g. small versus large) may reduce oddity by altering their foraging activities (Peuhkuri 1998), suggesting that other factors such as competition for food may also be involved in the decision to shoal with individuals of particular sizes.

However, predation is not the only factor that affects larval survival in the field. While a predator's decision to attack a particular prey is influenced by the immediate nutritional returns, the decision for parasitoids will be influenced by the potential longer-term nutritional value for her offspring. Parasitoids may select hosts depending on their size, nutritional content and ease of digestability (Elgar & Jebb 1999; Costa et al. 2004) and use a combination of olfactory and visual cues to locate hosts (Köpf et al. 1997; Stireman 2002; Depalo et al. 2010; Ichiki et al. 2011; Depalo et al. 2012; Bora & Deka 2014). As larger group size can increase visual detection risk by parasitoids (Low 2008), larger aggregations do not necessary increase individual survival in chemically defended prey (Lindstedt et al. 2011; Daly et al. 2012).

Most macrohabitats are thermally heterogeneous, and an animal can modify its temperature through activity and the choice or manipulation of their microhabitat (Huey 1991; Anne & Rasa 1995; Ruf & Fiedler 2000; Ohashi et al. 2005; De Los Santos et al. 2006). Unsurprisingly, gregarious behaviour increases the temperature of individuals across a range of species (Villa & Rinderer 1993; Klok & Chown 1999; Ruf & Fiedler 2000; Fletcher 2009). This has implications for larval growth, as heat gains may reduce developmental time (Ruf & Fiedler 2000; Ronnås et al. 2010; Aubernon et al. 2016) and improve immune responses (Catalán et al. 2012), ultimately improving survival (Wade & Breden 1986; Ronnås et al. 2010; Aubernon et al. 2016). Larval aggregation also improve establishment and feeding efficacy, particularly in younger instars (Ghent 1960; Tsubaki 1981; Weed 2010). Increased group size can have a positive effect on the size attained (Costa & Ross 2003), which is positively correlated to adult size at emergence (Bartlett & Ashworth 1988; Smith 2002) and larger sizes are generally associated with higher fecundity (Bartlett & Ashworth 1988; Tammaru et al. 1996) and lower mortality (Smith 2002).

Aggregations of paropsine leaf beetle larvae (Coleoptera, Chrysomelidae) of different ages and species have been documented for decades (Cumpston 1939; Carne 1966; Grégoire 1988). The adults and larvae of two species of Australian leaf beetles, Paropsis atomaria and Paropsisterna variicollis, occur together on the same Eucalyptus trees over spring and summer (Cumpston 1939; Mo & Farrow 1993). The larvae of both species may aggregate through random processes, or they could be driven by similar preferences for microhabitats, thus resulting in a shared space. As the nutrition content of leaves declines with leaf maturation numerous species, including Eucalyptus (Schultz et al. 1982; Coley 1983; Ayres & MacLean 1987; Wendler et al. 1995; Diaz et al. 2008; Balemi 2011), most herbivorous insects, including of P. atomaria and Pa. variicollis, prefer young leaves (Cumpston 1939; Gherlenda et al. 2016). The aposematic colouration of these larvae is viewed as protection against vertebrate predators (Moore 1967; Matthews & Reid 2002), but evidence is limited (Elek 2012). The larvae of P. atomaria and Pa. variicollis have contrasting colour patterns of yellow and black that vary according to instar. First instars are minute and yellowish but with many small black spots and black ends of the body. Second and third instars are yellow, with a black head and abdominal apex (hereafter referred to as the 'early instar colour pattern'), and the fourth instars of both species additionally have a black prothorax and three black dorsal and lateral abdominal stripes ('late instar colour pattern') (Cumpston 1939). In addition to similar colour patterns, the larvae of both species respond similarly to disturbance by rearing up their abdominal apices and everting a pair of glands. These glands release volatile secretions that include hydrogen cyanide, benzaldehyde and glucose (Moore 1967). Cyanide is toxic, largely due to the inhibition of cytochrome oxidase-producing cytotoxic anoxia (Chen & Rose 1952). Moore (1967) observed that Iridomyrmex ants, potential predators found naturally in the same habitat, were usually dead within 30 mins of coming into contact with the secretions of P. atomaria larvae. Disturbed larvae also regurgitate and defecate digested Eucalyptus leaves, containing anti-herbivore metabolites such as terpenoids and phenolics (Brophy et al. 1991; Batish et al. 2008).

Paropsine larvae have a number of invertebrate enemies, including tachinid and hymenopteran parasitoids (Cumpston 1939; Tanton & Khan 1978; Tanton & Epila 1984a; Selman 1994), and predatory coccinellid beetles and bugs (Tanton & Khan 1978). Tachinids are the predominant parasitoids of chrysomelines in Australia and lay eggs on the cuticle of host larvae, from which the parasitoid larvae subsequently emerge and burrow into the host body (Colless 2012). Tachinids are not strongly host-specific, attacking all similar eucalypt leaf feeding beetle larvae, including the weevil genus Gonipterus. The presence of hymenopteran parasites is not externally visible but these are either hyperparasitoids of tachinids or form a small percentage of larval parasitoids (Tanton & Epila 1984b).

The existence of mixed age groups and heterospecific aggregations of P. atomaria and Pa. variicollis is puzzling, because of the different signals contributed by larvae of different instars. A critical issue is whether the benefits of being in an aggregation outweigh the costs of presenting conflicting signals to potential predators. Field experiments reveal that larval models without stripes were preyed on more heavily than models with stripes (Tan et al. 2016), suggesting that i) the survival of larvae with the early instar colour pattern will be compromised when they are in the presence of other larvae with the late instar colour patterns, and ii) ‘odd’ individuals in an aggregation will suffer a greater predation rate. Evaluating these predictions is challenging, and our approach was to consider how larval survival prospects might vary according to the composition of the aggregation.

This study aims to determine if environmental preferences contribute to the heterospecific aggregations, and the potential costs for larvae of living in heterospecific aggregations. We hypothesized that the two species may have similar environmental preferences, and heterospecific aggregations may be driven by preferences for similar habitats. We surveyed natural aggregations of the larvae of both species in the field, and recorded environmental variables of these aggregations. Next, we conducted field experiments of larvae to investigate the survival and gregarious behaviour of larvae in heterospecific aggregations. We hypothesized that i) the survival of larvae with the early instar colour pattern will be compromised when they are in the presence of other larvae with the late instar colour patterns, and ii) gregarious behaviour of the larvae should be similar across aggregation types (monospecific and heterospecific), in order for heterospecific aggregations to persist.

Materials and methods

The observations and experiments took place at a Eucalyptus plantation at the Koala Conservation Centre, Phillip Island, Australia (38°29'6.6"S, 145°15'39.8"E).

Habitat preference

During November-December 2012, we surveyed aggregations of larvae on the eucalypt trees, and recorded corresponding environmental variables: ambient light at the aggregation, leaf surface temperature, ambient temperature and height from ground. Eucalyptus trees were systematically surveyed from ground up to a height of 1.90 m, for the presence of larvae. The observed aggregations were categorised based on the species composition – P. atomaria, Pa. variicollis or heterospecific aggregation; and the colour patterns of the larvae – early or late instar, or a mixture of both. Paropsis atomaria larvae have yellow legs while Pa. variicollis larvae have black legs, and this feature was used to distinguish between the species. Legs are mostly hidden under the larval body, so this colour difference is not likely to be a significant part of the aposematic system. We performed the Shapiro-Wilk test to test for normality in the distribution of aggregation sizes. As the aggregation sizes across the three aggregation types were not normally distributed, we performed Kruskal-Wallis tests to examine if the distribution of aggregation sizes was different across the three aggregation types, and a post-hoc Wilcoxon test with Bonferroni correction to examine differences between the aggregation types. Next, we examined if the number of larvae of P. atomaria and Pa. variicollis in heterospecific groups was correlated. Using Fisher’s exact test, we examined if species composition (P. atomaria, Pa. variicollis, heterospecific) had an effect on the colour patterns of aggregations observed.

In contrast to many cryptic species, mimics and models may be conspicuous by staying on the upper surfaces of leaves rather than underneath (Rettenmeyer 1970). Thus, we recorded the location of the larval aggregations (under the leaf, on top of the leaf, on leaves that were sideways, or on the petiole) to estimate the visibility of the larval aggregations by visually seeking predators. We analysed the effects of group size, position of larvae and environmental variables – ambient temperature, leaf surface temperature, ambient light and height from ground – on the species composition of naturally occurring larval aggregations using multinomial logistic regressions. The initial model with all the interactions was explored, and non-significant ($P > 0.05$) interaction terms were subsequently dropped from the model and not reported. We performed multinomial logistic regressions using the nnet package (Venables & Ripley 2002) and the analysis of deviance using the car package (Fox & Weisberg 2011). Models were visualised using the effects package (Fox 2003; Fox & Hong 2009). All analyses were performed in R (R Development Core Team 2016).

Gregarious behaviour and survival of larvae in heterospecific aggregations

We collected larvae of P. atomaria and Pa. variicollis from trees in the plantation and used them in four treatments that varied in both species and colour patterns. These included monospecific treatments of P. atomaria and Pa. variicollis, which were used to compare the gregarious behaviour and the survival of larvae in heterospecific aggregations. Two heterospecific treatments included aggregations in which both species had either similar or different colour patterns.

Each treatment comprised ten individuals drawn from two ‘sets’ of five larvae from a naturally existing aggregation. The four treatments had the following combinations of larval ‘sets’: I) two sets of third instar P. atomaria larvae from different aggregations; II) two sets of fourth instar Pa. variicollis larvae from different aggregations; III) one set of third instar P. atomaria larvae and one set of third instar Pa. variicollis larvae; IV) one set of third instar P. atomaria larvae and one set of fourth instar Pa. variicollis larvae. We selected P. atomaria larvae that were of similar length to the larvae of Pa. variicollis in each treatment. A total of 10 replicates involving 100 larvae were performed for all four treatments. However, after the first day of the experiment, larvae of two replicates for treatment I, and one replicate for treatment III had disappeared and the data for these replicates were disregarded. We predicted no differences in the survival of larvae of P. atomaria and Pa. variicollis when they had similar colour patterns (i.e. third instar of Pa. variicollis species, and second instar of P. atomaria species). However, we predicted differences in survival when the larvae had different markings: following (Tan et al. 2016), fourth instar larvae of Pa. variicollis larvae with black stripes should suffer lower predation than the third instar larvae of P. atomaria.

The groups of larvae for each treatment were placed on Eucalyptus saplings that had been previously cleared of all other externally feeding larvae. Björkman et al. (2000) suggested that the complexity of plant architecture can play a role in the predation rates of heteropterans, but all the plants used in this study across the treatments were Eucalyptus saplings with similar, simple architecture. Paropsine larvae feed on immature leaves (Carne 1966; Ohmart & Edwards 1991), so the choice of saplings simulates the natural microhabitats of the two species. Eggs of both beetle species are typically oviposited on or near the terminal shoots (Cumpston 1939; Carne 1966), and the larvae were relocated onto sub-terminal leaves for the experiment. The presence and behaviour of the larvae on the saplings was observed an hour after their introduction, and at 0900 hours, 1300 hours and 1700 hours daily for the following four days. Instances of predation, presence of potential predators and parasitic eggs were recorded during these observations. Predators such as heteropterans can be a major source of larval mortality in chrysomelids (Phillips 1977; Björkman et al. 2000; Björkman et al. 2003)(**Fig. S1a**). Our observations of predation events may underestimate the true rate of predation, as they are derived from point sampling. However, we used this method as it causes minimal disruption to the fauna and thus, potential predators. Parasitoidism of the larvae was recorded as the number of larvae with tachinid eggs, which were clearly visible (**Fig. S1b**). Prior parasitoid attacks on the larvae before commencement of the experiment would not affect the results because ovipositing tachinid females apparently do not discriminate against previously attacked host individuals (Belshaw 1994).

Analysis of gregarious behaviour and larval survival

The gregarious behaviour of larvae was scored to ascertain how likely they were to be in a monospecific or heterospecific group. Larvae were considered to be part of a group when they were in physical contact with another individual as described in Cumpston (1939). The overall gregarious behaviour of larvae was scored as a function of the number of larvae (i) observed in aggregations and the number of neighbours each larva had (i - 1). For j number of aggregations for a total of k larvae, the overall gregarious score was:

$$\text{Overall gregarious score} = \frac{i_1(i_1-1)+i_2(i_2-1)+\cdots i_j(i_j-1)}{k}$$

Monospecific gregarious scores were calculated in the same way as the overall gregarious score.

A measure of the gregarious behaviour of each species in heterospecific treatments (III and IV) was estimated as:

$$\text{Heterospecific gregarious score} = \frac{l_1/i_1+l_2/i_2+\cdots l_j/i_j}{m}$$

where l is the number of like-species neighbours in each aggregation of i larvae, and m is the total number of like-species larvae present in j aggregations. The gregarious scores were then square root-transformed to improve the distribution of the data.

A generalised linear model was used to determine the effect of treatment and day on the gregarious behaviour of the larvae. The initial full model with interactions of treatment and day with the gregarious scores was explored, and non-significant ($P > 0.05$) interaction terms subsequently removed. Non-significant variables that did not have significant interaction terms were also removed and the reduced model only is reported. A generalised linear model was also used to determine the effect of P. atomaria and Pa. variicollis monospecific gregarious score, treatment type and day on heterospecific gregarious score. The initial model with interactions of the heterospecific behaviour scores with all of the above factors was explored, and non-significant ($P < 0.05$) interaction terms subsequently removed. As above, non-significant variables that did not have significant interaction terms were also removed and the reduced model only is reported.

Survival analysis of the larvae was performed in R, using the Survival package (Therneau 2013). The survival of larvae was compared across treatments.

Results

Habitat preference

We observed a total of 3041 larvae in 312 aggregations: 150 aggregations of P. atomaria comprising 563 individuals; 133 aggregations of Pa. variicollis comprising 2085 individuals, and a total of 393 larvae in 29 heterospecific aggregations of P. atomaria (n = 134) and Pa. variicollis (n = 261). 23.8% of the total number of P. atomaria larvae surveyed was found in heterospecific aggregations, compared with 12.5% of Pa. variicollis larvae. **Fig. 1** shows the group size of naturally found aggregations with respect to species composition. A Kruskal-Wallis test revealed a significant effect of aggregation type on the number of larvae (X^2_2= 105.7, P <0.001). A post-hoc Wilcoxon test with Bonferroni correction showed that P. atomaria aggregations were significantly smaller than that of Pa. variicollis aggregations (P <0.001) and heterospecific aggregations (P <0.001). The number of larvae of P. atomaria and Pa. variicollis in heterospecific groups was not correlated (R^2 = <0.001, P = 0.98, df = 27). The observed colour patterns of aggregations depended upon species composition (P. atomaria, Pa. variicollis, heterospecific) (P < 0.001; **Fig. 2**). Heterospecific aggregations had less variation in patterns compared with monospecific P. atomaria or Pa. variicollis aggregations.

The gregarious behaviour of larvae at rest (i.e. larvae that were not feeding) varied between species: P. atomaria larvae grouped in a compact series of overlapping rows, resting longitudinally on the leaf, with the heads of larvae in each row touching the ventral thoracic regions of anterior larvae, while Pa. variicollis larvae arranged themselves on the leaf in a circular fashion, overlapping when numerous, with their heads pointed towards a central focus, i.e. cycloalexy (Weinstein 1989). Larvae in heterospecific aggregations tended to arrange themselves in either fashion (**Fig. 3**).

The mean environmental parameters for aggregations, classified according to their species composition, are listed in Table 1. Multinomial logistic regression of the effects on species composition showed that the position of the larval aggregation, leaf surface temperature, ambient light, and group size were significantly different across the species composition types (Table 2, Fig. 4). Heterospecific aggregations occurred in relatively less visually conspicuous locations overall. Monospecific aggregations of P. atomaria and Pa. variicollis were more likely to occur on the petioles, compared to heterospecific aggregations (Fig. 4a). Heterospecific aggregations were less likely to occur in brighter ambient light conditions (Fig. 4b). While monospecific aggregations of P. atomaria and Pa. variicollis were more likely to occur at lower leaf temperatures, heterospecific aggregations were more likely to occur at higher leaf temperatures (Fig. 4c). Pa. variicollis and heterospecific aggregations were more likely to be larger than P. atomaria aggregations (Fig. 4d). The height of larval aggregation from the ground and ambient temperature did not have a significant effect on species composition (Table 2, Fig. S2). Further details of the model are reported in Table 2.

Gregarious behaviour and survival of larvae in heterospecific aggregations

A total of 10 replicates involving 100 larvae were included in each of four treatments. After the first day of the experiment, larvae of two replicates for treatment I, and one replicate for treatment III had disappeared and the data for these replicates were disregarded in the analyses, leaving eight replicates for treatment I, ten replicates for treatment II, nine replicates for treatment III and ten replicates for treatment IV. The larval aggregation behaviour ranged from solitary individuals to monospecific and heterospecific aggregations. There was no difference in the overall gregarious behaviour of larvae across the four treatments ($F_{3,176} = 2.034$, $P = 0.111$) but there was an effect of day on the overall gregarious behaviour ($F_{4,179} = 6.084$, $P < 0.001$;). Post-hoc comparisons using the Tukey HSD test indicated that day 2 had significantly higher gregarious behaviour than days 4 and 5 (Table 3).

The overall and heterospecific gregarious scores of P. atomaria and Pa. variicollis larvae did not indicate that the larvae behaved differently in heterospecific compared with monospecific aggregations. There was no difference in the overall and heterospecific gregarious behaviour (measured by gregarious scores) of P. atomaria or Pa. variicollis larvae in heterospecific aggregations in days 1, 2 and 4 (P. atomaria, day 1: $F_{1,18} = 1.10$, P = 0.31; day 2: $F_{1,18} = 0.44$, P = 0.52; day 4: $F_{1,18} = 3.68$, P = 0.07; Pa. variicollis, day 1: $F_{1,18} = 2.69$, P = 0.12; day 2: $F_{1,18} = 0.11$, P = 0.75; day 4: $F_{1,18} = 1.80$, P = 0.20). However, the gregarious behaviour of P. atomaria larvae on days 3 and 5 were significantly different from Pa variicollis. On day 3, the overall gregarious behaviour of heterospecific groups was higher when P. atomaria larvae were more gregarious (Day 3: $F_{1,18} = 7.58$, P = 0.019; Day 4: $F_{1,18} = 11.62$, P = 0.004). There were also interaction effects on these days: on Day 3 between gregarious behaviour of P. atomaria and Pa. variicollis and the treatment type ($F_{1,18} = 7.60$, P = 0.019), and on day 5 between the gregarious behaviour of P. atomaria and the treatment type ($F_{1,18} = 6.34$, P = 0.025).

The survival of larvae did not significantly differ across the treatments ($X^2_3 = 4.80$, P = 0.19; **Fig. 5a**), nor was there a significant interaction effect between treatment and species. Pa. variicollis larvae had a higher survival rate (mean = 0.485) than P. atomaria larvae (mean = 0.347). The survival of P. atomaria did not differ significantly across the treatments ($X^2_2 = 2.95$, P = 0.23; **Fig. 5b**). The survival of Pa. variicollis larvae was not comparable across treatments III and IV as the larvae in the two treatments were of different instars. For treatments II and IV where Pa. variicollis larvae were in the fourth instar, the survival was not significantly different ($X^2_1 = 0.23$, P = 0.89).

Specific predation events were not recorded, but predators included the assassin bug, Gminatus sp. There was one observed instance of predation in each of treatments I, II and IV, while three instances of predation were observed for treatment III. Parasitoidism rates by tachinid flies depended on the treatment and species. The parasitoidism rate on Pa. variicollis larvae was similar throughout the treatments, while that of *P. atomaria* varied depending on treatment (Table 4). However, the instances of parasitoidism are too few for formal statistical analysis.

Discussion

Our survey of natural aggregations suggest that environmental preferences contribute to the heterospecific aggregations, with heterospecific aggregations occurring more commonly at higher leaf temperatures, and in less visually conspicuous locations. Manipulation of larval aggregations in the field indicate that predation rates and survival of larvae did not significantly differ across heterospecific and monospecific aggregations, providing no evidence for the 'oddity effect' in the heterospecific aggregations of mixed phenotypes, or that 'odd' individuals confuse potential predators.

There may be other benefits driving aggregation that we did not measure in our study. For instance, animals in aggregations experience lower water loss compared with solitary individuals (Klok & Chown 1999). However, evaporative water loss is unlikely to be a major factor favouring aggregationg behaviour, since the larvae in our study are transpiring in an open space in the field, and individual transpiration is unlikely to affect the surrounding relative humidity of adjacent aggregating individuals.

Larval survival was not influenced by colour pattern composition (treatment IV), which is surprising because previous experiments with larval models indicated that between individual differences in signals increased mortality rates (Tan et al. 2016). Presumably, the persistence of heterospecific groups in these two species of chrysomeline larvae reflects the benefits of aggregating outweighing the costs of presenting diverse signals to potential predators. Heterospecific aggregations may be driven by environmental preferences. As poor leaf nutrition content can negatively affect larval growth (Ayres & MacLean 1987), the preference of P. atomaria and Pa. variicollis larvae for young Eucalyptus leaves may predispose the larvae to exploit similar microhabitats. The benefits of larval aggregations in establishment, feeding efficacy and development (Ghent 1960; Tsubaki 1981; Wade & Breden 1986; Ronnås et al. 2010; Weed 2010; Catalán et al. 2012; Aubernon et al. 2016) may further favour heterospecific aggregations, leading to similar gregarious behaviour of both species of larvae across monospecific and heterospecific aggregations. Avian predators typically learn to avoid aposematic prey more rapidly and more strongly if they are aggregated rather than solitary (Gagliardo & Guilford 1993; Alatalo & Mappes 1996; Gamberale & Tullberg 1996; Riipi et al. 2001). The costs of presenting diverse signals may be mitigated by the preference of larvae in natural heterospecific aggregations for less visually conspicuous locations, such as leaf petioles, even at higher temperatures. Preference for particular habitats according to species composition in groups could be responsible for the lack of difference in survival.

Studies in aposematic colouration of gregarious animals predominantly focus on the effects of visually seeking vertebrate predators (Gagliardo & Guilford 1993; Alatalo & Mappes 1996; Gamberale & Tullberg 1996; Alatalo & Mappes 2000). Our study highlights the potential significance of parasitoids as a selection pressure acting on aposematic colouration: both P. atomaria and Pa. variicollis are hosts to several parasitoid species (Tanton & Khan 1978; Tanton & Epila 1984b; Colless 2012), and although parasitoidism rates in this study were lower than those observed elsewhere (Tanton & Epila 1984b), the brief timeframe of our experiments may underestimate the true rates. Parasitoidism by tachinid flies in particular may be a significant force shaping the coexistence of heterospecific aggregations comprising P. atomaria and Pa. variicollis, because parasitoids can influence the coexistence of different host species ((Renaud et al. 1996). Our unexpected finding that parasitoidism rates on monospecific larval aggregations of P. atomaria was two to four times that of larvae in heterospecific treatments is consistent with this view. Perhaps heterospecific aggregations arise from the preference of P. atomaria larvae to aggregate with Pa. variicollis: Pa. variicollis and heterospecific aggregations were typically larger than P. atomaria aggregations, and heterospecific aggregations were more likely to occur at higher leaf temperatures that that for monospecific aggregations of both species. Individuals of P. atomaria, by joining aggregations of Pa. variicollis, may reduce the risk of parasitoid attacks, especially when they are more active, such as during higher temperatures (López et al. 1997).

If large host size benefits parasitoid larval development (Stenberg 2012; Stenberg 2015), then tachinid parasitoids may favour P. atomaria larvae over Pa. variicollis larvae because the former are larger (Reid 1992). Tachinid parasitoids use a combination of olfactory and visual cues to locate hosts (Stireman 2002; Depalo et al. 2010; Ichiki et al. 2011; Depalo et al. 2012; Bora & Deka 2014), and while tachinids target a variety of lepidopterans, aposematic hosts tend to support less diverse tachinid assemblages (Stireman & Singer 2003). Nevertheless, defensive larval secretions may act differently on parasitoids compared with predators. Tachinid larvae burrow into the host body after hatching (Colless 2012), and the growing tachinid larvae may evade the chemical defences of the host larvae by avoiding ingestion of the glands containing the defensive secretions. On the other hand, predators may not be able to avoid ingesting the defensive glands if the chrysomeline larvae are swallowed whole.

Closely related species are usually more phenotypically different when in sympatry because of ecological character displacement (Brown & Wilson 1956). As competition for resources leads to divergence in resource use or acquisition (Schulter et al. 1985), sympatric species that exist in heterospecific aggregations present opportunities to examine the mechanisms that drive the association (Landeau & Terborgh 1986). Similarities in colour pattern can be the result of (i) convergent evolution; (ii) parallel evolution through Müllerian advantage; or (iii) parallel evolution without Müllerian advantage (Brower et al. 1963). It is possible that the similar colour patterns in the larvae of P. atomaria and Pa. variicollis represent Müllerian mimicry, as both species are unpalatable; both are found at the same place and time; and they are not distinguished by predators. Heterospecific aggregations of the same instars of P. atomaria and Pa. variicollis could arise in response to intense predation, which then selects for a Müllerian advantage.

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Fig. 1 Bar plot of mean group size of larvae in monospecific and heterospecific aggregations with standard error values reflected in the error bars

Fig. 2 Frequency of colour patterns observed in aggregations of larvae. The grey areas represent aggregations with early instar colour pattern; open areas represent aggregations with late instar colour pattern; black areas represent aggregations with mixed instar colour pattern.

Fig. 3 Paropsis atomaria and Paropsisterna variicollis larvae in the field. a) Monospecific aggregation of P. atomaria; b) Monospecific aggregation of Pa. variicollis; c) Heterospecific aggregation with larval arrangement after that of P. atomaria; d) Heterospecific aggregation with larval arrangement after that of Pa. variicollis. White spots indicate P. atomaria larvae and the white bar in each image represents 5mm.

Fig. 4 Visualisation of multinomial logistic regression model of the effects of variables. a. Larval position on leaf (P: on petiole; S: on side of leaf; T: on top of leaf; U: under leaf); b. ambient light environment measured in lux; c. leaf surface temperature (°C); d. number of larvae, on the species composition of larval aggregations. The y-axis represents the probability of each aggregation type (P. atomaria, Pa. variicollis, or heterospecific aggregations) occurring for each variable. Grey areas in graphs b-c indicate the standard deviation, while the solid line indicate the mean.

Fig. 5 Kaplan-Meier survival curves of larvae across treatments. A. Overall survival of larvae was not different across the four treatments ($X^2_3 = 4.80$, $p = 0.19$). B. Survival of P. atomaria was not different across the three treatments ($X^2_2 = 2.95$, $p = 0.23$). Treatment I: n = 8; Treatment II: n = 10; Treatment III: n = 9; Treatment IV: n = 10. The number of larvae at the start of each replicate was 10.

Table 1 Environmental parameters based on aggregation composition with standard error values

Species composition	Ambient light (lux)	Ambient (°C)	Height from ground (m)	Leaf surface temperature (°C)
P. atomaria	36.50 ± 7.37	23.40 ± 2.91	1.29 ± 0.28	14.44 ± 0.16
Pa. variicollis	34.70 ± 7.66	23.03 ± 3.08	1.36 ± 0.25	13.76 ± 0.26
Heterospecific aggregations	33.05 ± 10.54	22.37 ± 2.52	1.43 ± 0.30	16.10 ± 0.47

Table 2 Multinomial logistic regression of the effects on larval aggregation composition (P. atomaria, Pa. variicollis, or heterospecific aggregations)

Variable(s)	df	X^2	P
Ambient light	8.43	2	0.015
Ambient temperature	1.97	2	0.37
Group size	63.38	2	<0.001
Height from ground	3.25	2	0.20
Leaf surface temperature	14.63	2	<0.001
Position on leaf	16.15	6	0.013
Ambient light*leaf temperature*ambient temperature	24.60	2	<0.001
Height*ambient temperature*group size	8.99	2	0.011
Height*leaf temperature*ambient temperature	7.96	2	0.019
Leaf temperature*ambient light*position*group size	13.88	6	0.031
Height*leaf temperature*ambient light*ambient temperature *group size	26.61	2	<0.001

Table 3 Mean overall gregarious scores of the four treatments with standard error mean values values

Treatment	n	Day				
		1	2	3	4	5
I	8	1.80 ± 0.33	1.80 ± 0.18	1.26 ± 0.30	1.03 ± 0.25	1.05 ± 0.26
II	10	1.67 ± 0.17	2.23 ± 0.23	1.94 ± 0.28	1.48 ± 0.21	1.20 ± 0.25
III	9	1.51 ± 0.20	1.78 ± 0.11	1.59 ± 0.22	1.15 ± 0.29	1.28 ± 0.27
IV	10	1.18 ± 0.20	1.81 ± 0.21	1.51 ± 0.24	1.39 ± 0.22	1.29 ± 0.14

Table 4 Parasitoidism rates of larvae across treatments

Treatment	Larval species	No. of larvae	No. of larvae parasitised	Parasitoidism rate (%)
I	*P. atomaria*	78	7	8.97
II	*Pa. variicollis*	100	2	2.00
III	*P. atomaria*	45	2	4.44
	Pa. variicollis	46	1	2.17
IV	*P. atomaria*	48	1	2.08
	Pa. variicollis	49	1	2.04

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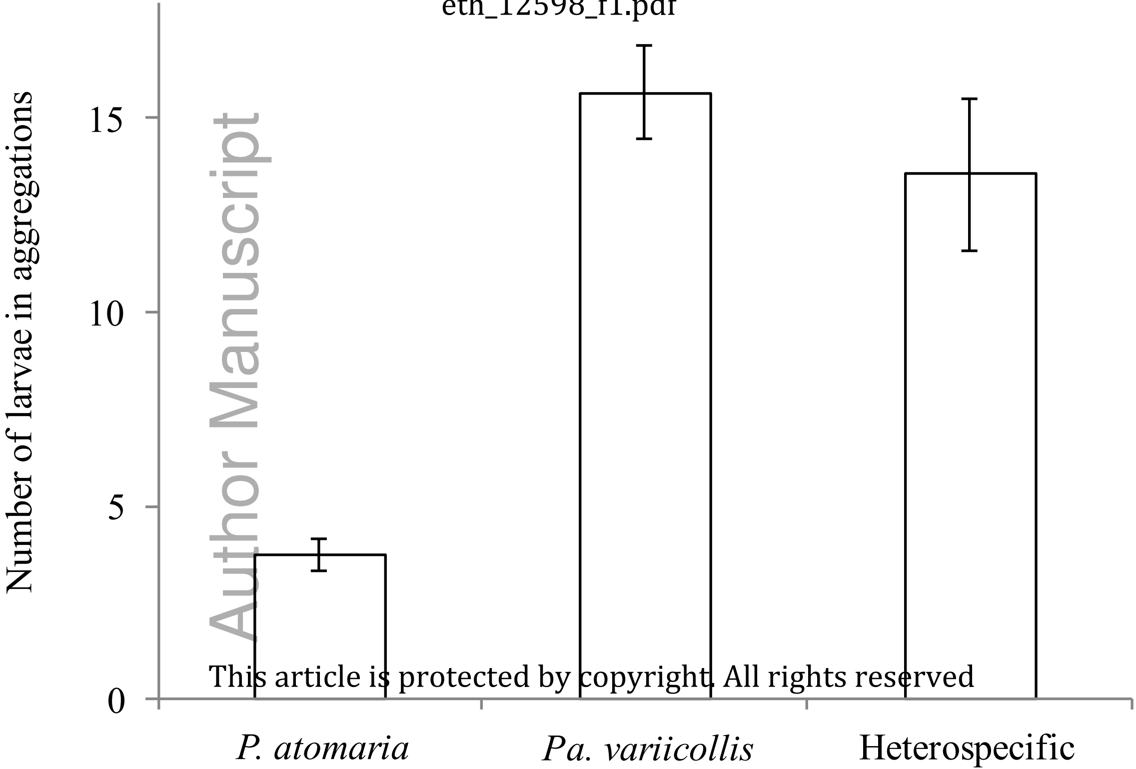
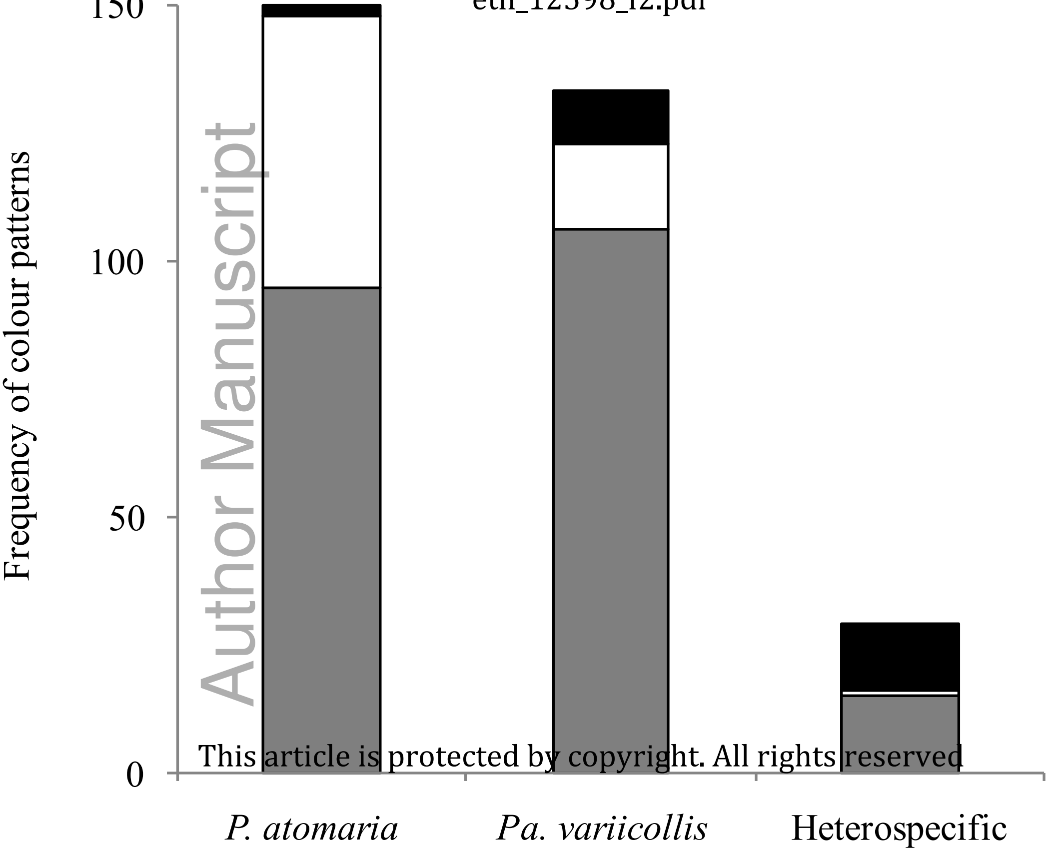
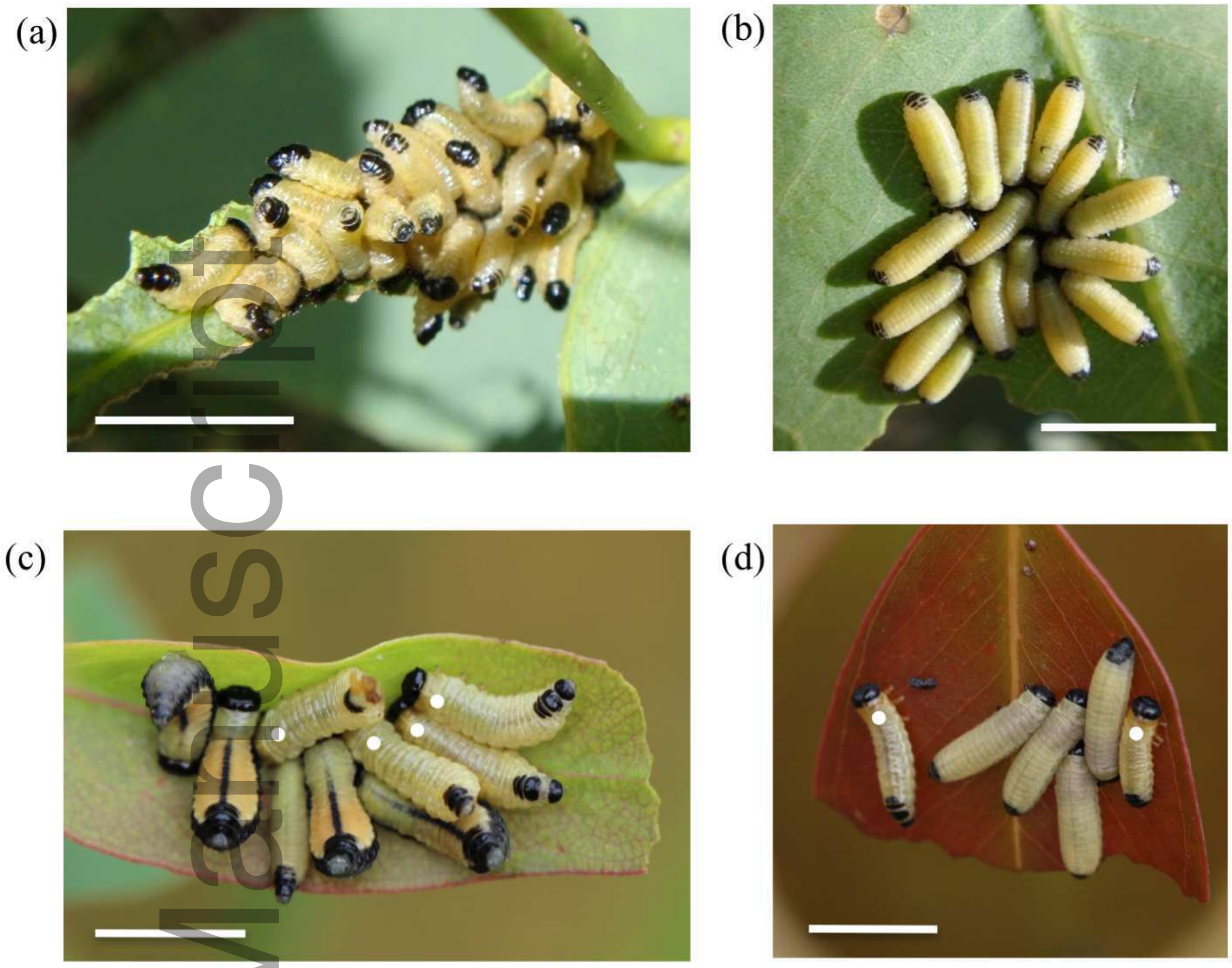
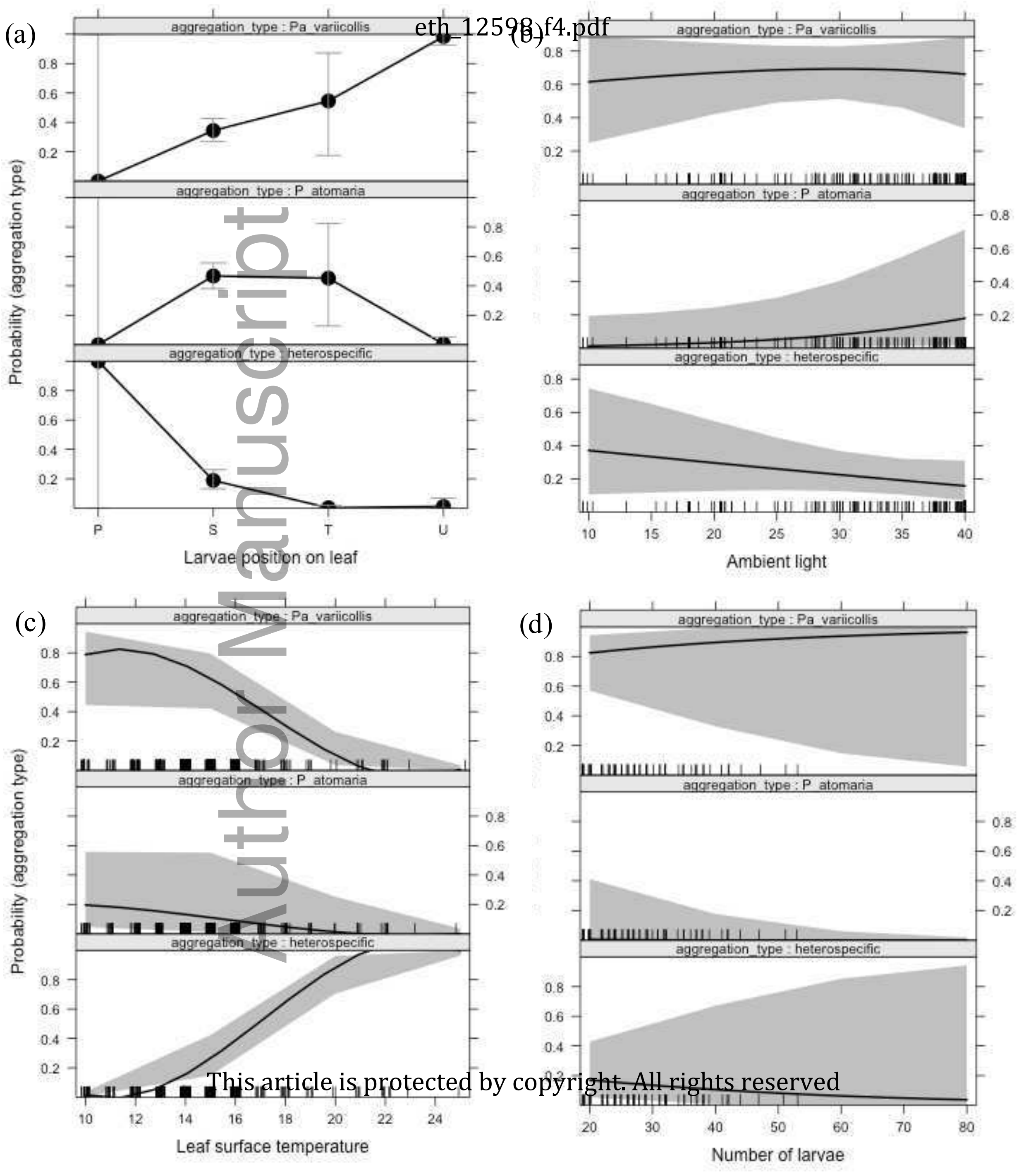



Frequency of colour patterns
150
100
50
0
P. atomaria
Pa. variicollis
Heterospecific


(a)
(b)
(c)
(d)


(a)
aggregation_type : Pa_variicollis
aggregation_type : P_atomaria
aggregation_type : heterospecific
Probability (aggregation type)
0.2
0.4
0.6
0.8
P
S
T
U
Larvae position on leaf
(b)
aggregation_type : Pa_variicollis
aggregation_type : P_atomaria
aggregation_type : heterospecific
10
15
20
25
30
35
40
Ambient light
(c)
aggregation_type : Pa_variicollis
aggregation_type : P_atomaria
aggregation_type : heterospecific
Probability (aggregation type)
10
12
14
16
18
20
22
24
Leaf surface temperature
(d)
aggregation_type : Pa_variicollis
aggregation_type : P_atomaria
aggregation_type : heterospecific
20
30
40
50
60
70
80
Number of larvae

eth_12598_f5.pdf