

Variation in social and sexual behaviour in four species of aposematic seed bugs (Hemiptera: Lygaeidae): The role of toxic and non-toxic food



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ABSTRACT

Understanding variation in social behaviour both within and among species continues to be a challenge. Evolutionary or ecological theory typically predicts the optimal behaviour for an animal under a given set of circumstances, yet the real world presents much greater variation in behaviour than predicted. This variation is apparent in many social and sexual interactions, including mate choice, and has led to a renewed focus on individual variation in behaviour. Here we explore within and among species variation in social behaviour in four species of aposematic seed bug (Lygaeidae: Hemiptera). These species are *Müllerian mimics*, with characteristic warning colouration advertising their chemical toxicity. We examine the role of diet in generating variation in two key behaviours: social aggregation of nymphs and mate choice. We test how behaviour varies with exposure to either milkweed (a source of defensive compounds) or sunflower (that provides no defence). We show that although the four species vary in their food preferences, and diet influences their life-history (as highlighted by body size), social aggregation and mate choice is relatively unaffected by diet. We discuss our findings in terms of the evolution of aposematism, the importance of automimicry, and the role of diet in generating behavioural variation.

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1. Introduction

Social interactions are key components of fitness. These range from potentially brief interactions between males and females associated with mating, through to animals that aggregate either temporarily or in the form of longer term groups (including overwintering assemblages and colonies) (Bleakley et al., 2010). Such interactions often vary over the course of an animal's life, but the factors that cause variation in social behaviours, both within and between species, are not yet fully understood (Ebensperger et al., 2012). For instance, there has been a lot of attention given to variation in mate choice (reviewed by Jennions and Petrie, 1997; Bateson and Healy, 2005), including the extent to which mating outcomes are repeatable (e.g. Shuker and Day, 2002). Despite this attention, it is still unclear whether such variation is adaptive, and the extent to which the variation we see is explained by factors such as learning, local ecology, or chance. One problem is that much of the theoretical machinery we have for predicting the outcomes of social behaviour seeks to provide an optimal behaviour for a given set of circumstances (e.g. the classic behavioural ecological approach: Davies

et al., 2012). Whilst this approach has been enormously successful, such models are often not geared towards predicting (and explaining) among-individual variation in behaviour (Sih et al., 2012). This has led to a renewed focus on understanding individual variation in behaviour more generally (including investigation of the related concepts of animal personalities and behavioural syndromes: (e.g. Pruitt and Ferrari, 2011; Pruitt et al., 2012; Bell, 2012; Sih et al., 2012; Sih and Del Giudice, 2012)).

In this paper we consider the effects of diet, focusing on aposematic insects that obtain chemical defence from their food, and explore how variation in diet influences variation in behaviour. Aposematic species display their chemical defences using warning colours, such as red and black, or yellow and black (Ruxton et al., 2004). Additionally other signals may be used, such as chemical cues (Aldrich, 1988), sounds or distinctive behaviours (De Wert et al., 2012). These cues signal to potential predators that the organism is toxic or distasteful. In order for such signals to be successful, predators must learn to associate them with unpalatability or toxicity, and so there is selection favouring members of the same population that exhibit the same signals. That way, once a predator has encountered one individual of an aposematic species it will, theoretically at least, avoid all members of that species (Ruxton et al., 2004). However, not all aposematically coloured animals are defended, as non-defended species may mimic the markings of

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defended ones in order to take advantage of predator's reluctance to attack potentially toxic prey (i.e. Batesian mimicry: (Ruxton et al., 2004). This contrasts with Müllerian mimicry, when two or more protected species evolve similar markings, sharing the costs of predator education and benefiting from predators generalising from one toxic prey species to another (Tullberg et al., 2000). However, even among truly distasteful species, not all individuals may be chemically defended or the levels of chemical protection may not be uniform across all individuals in a species, or even a population (Speed et al., 2012). This is especially true for animals that sequester their protective chemicals from their food (Bowers, 1992). This "automimicry" may arise from individuals being unable to find toxic defensive compounds to sequester, or as a "cheating" strategy by which undefended individuals do not pay the cost of being chemically defended (Speed et al., 2012; Holen and Svenningsen, 2012). Here, we consider the extent to which social and sexual behaviour in four species of aposematic seed bug varies with access to a diet that either does or does not provide compounds used for chemical defence.

We focus on two behaviours. First, aposematic animals often appear to behave in ways that promote the effectiveness of their warning colouration, for instance by aggregating together as nymphs or larvae. For example, nymphs of the seed bug *Lygaeus equestris* suffered fewer attacks from domestic chicks (*Gallus gallus domesticus*) when presented in groups than when presented alone (Tullberg et al., 2000). This behaviour is presumed to present a stronger "signal" to warn off potential predators or help predators associate aposematically-coloured prey with toxins/bad-taste (Sillen-Tullberg, 1988; Gamberale and Tullberg, 1998). Additionally, group size has been found to positively affect the survival in the wild of nymphs of another aposematic seed bug, *Oncopeltus fasciatus* (Ralph, 1976). However, recent work has shown that even undefended, non-aposematic individuals can benefit from lowered predation rates by aggregating with aposematic individuals (De Wert et al., 2012). It remains unclear though how chemical defence influences aggregation behaviour.

Second, mate choice remains an important component of organismal fitness (Andersson, 1994; Kokko et al., 2003; Kotiaho and Puurtinen, 2007). There has been recent interest in the role of nutrition in sexual selection and mate choice, from the perspectives of both the chooser and the chosen, across a range of organisms (e.g. ground crickets, Hall et al., 2008; house mice, Costello et al., 2009; wolf spiders, Lomborg and Toft, 2009; fruit flies, Fricke et al., 2010; Nile tilapia, Giaquinto et al., 2010; bluefin killifish, McGhee and Travis, 2011; and cockroaches, South et al., 2011). Nutrition is important in terms of understanding condition-dependence of ornaments, the role of life-history trade-offs in sexual selection, and the nature of the benefits that may accrue to the choosy sex. Choosiness may also vary depending on the condition of the choosing sex (Cotton, Small, and Pomiankowski 2006). As well as the potential for indirect benefits arising from mate choice (i.e. good genes or sexy sons: reviewed by Andersson, 1994; Koella, 1989; Bateson, 1983; Prokop et al., 2012) many species also receive direct benefits from matings (Arnqvist and Nilsson, 2000). These benefits can take the form of ejaculates that act as food (DiRienzo and Marshall, 2013) or as a source of water (Edvardsson, 2007), to the female or her offspring (Gwynne, 2008).

However, females may receive other benefits from ejaculates or nuptial gifts such as increased chemical protection against predation (Cothran et al., 2012). For example, it may be adaptive for individuals to mate with chemically well-defended partners, if those partners can transfer those chemical defences to their offspring (either via eggs or via the ejaculate). One of the most elegant examples concerns the moth *Utetheesia ornatrix*. This species sequesters pyrrolizidine alkaloids from food as larvae. Females can transfer this chemical protection to their offspring; however males

can also transfer additional pyrrolizidine alkaloids to the female as part of his sperm package. This has been shown to not only increase the amount of protection a female can pass on to her offspring, but also to increase the female's own protection against predators following mating (Gonzalez et al., 1999). Perhaps unsurprisingly, females show a marked preference for males with high pyrrolizidine alkaloid levels (Dussourd et al., 1991). A similar phenomenon has also been found in the beetle *Neopyrochroa flabellate* (Eisner et al., 1996a,b). With this in mind, we might expect environmental factors, such as diet or chemical protection, to affect the potential benefits females can gain from mating, and thus, female mating decisions.

The Lygaeidae (*Sensu stricto*) are a large family within the Heteroptera (Burdfield-Steel and Shuker, in prep.) that contain a number of aposematic species (Fig. 1). These species typically sequester protective toxic compounds such as cardiac glycosides from the plants they feed on and both adults and nymphs advertise this protection with bright red or orange markings (Aldrich, 1988). In this study we consider four species. First, *O. fasciatus*, the 'milkweed bug' is considered to be a milkweed specialist as it is typically found on the common milkweed *Asclepias syriaca* and other plants of the sub-family Asclepiadaceae (Dingle et al., 1980a,b). The cardiac glycosides that individuals of this species sequester from their food provide them with protection from predators (Sillen-Tullberg et al., 1982; Berenbaum and Miliczky, 1984; Scudder et al., 1986). Adults of *O. fasciatus* feeding on milkweed seeds have been shown to be distasteful to mantids (Berenbaum and Miliczky, 1984) and lizards (Feir and Suen, 1971). Similarly, *L. equestris*, its sister species *Lygaeus simulans*, and *Spilostethus pandurus* typically feed on asclepiads (Wheeler, 1983) and show evidence of chemical protection from predators (Sillen-Tullberg et al., 1982; Gamberale and Tullberg, 1996; Svadova et al., 2010; Tullberg et al., 2000). Additionally, in *S. pandurus* sequestered cardiac glycosides have been shown to be incorporated into defensive secretions produced in the scent glands (Abushama and Ahmed, 1976). This protection has been shown to be diet-dependent in *L. equestris* (Tullberg et al., 2000). Thus it may be that individuals feeding on plants other than asclepiads may sacrifice chemical protection in exchange for niche expansion. Despite this, these unprotected (or less protected) individuals may still benefit from their resemblance to protected conspecifics (Berenbaum and Miliczky, 1984). This automimicry may well be very common (Speed et al., 2012), especially in groups such as lygaeids where variation in food sources may strongly influence levels of chemical protection (Tullberg et al., 2000).

We will explore how exposure to toxic versus non-toxic seeds influences social and sexual behaviour in these four Lygaeid species. We will do this in a series of four experiments. First we consider the extent to which nymphs preferentially select toxic compound-bearing food. We allowed nymphs to feed on either non-toxic sunflower seeds (*Helianthus annuus*) or a mixture of sunflower seeds and toxic milkweed seeds (*Asclepias syriaca*) from hatching. We predict that given a choice between toxic and non-toxic seeds nymphs of all four species will prefer the toxic food. In our second experiment, we compare the aggregation behaviour of nymphs fed on the two different diets. We predict a greater tendency to aggregate in nymphs fed on a toxic diet as these are likely to have greater chemical defence and reinforce the aposematic signal for potential predators. Our third experiment examines the effect of the two different diets on body size in both sexes of adult bugs to look for any evidence of life-history trade-offs associated with consuming toxic versus non-toxic foods. Our final experiment will consider mating preferences of both males and females fed on either toxic or non-toxic diets. We predict that female bugs will prefer to mate with males possessing greater chemical defences; alternatively there may be a preference in both sexes for bugs that were fed on the same food. Importantly our design allows us to test whether any



Fig. 1. Photos of the four Lygaeidae species used in this study. Clock wise from top left: *Lygaeus simulans*, *Lygaeus equestris*, *Oncopeltus fasciatus* (photo courtesy of Alison Bockoven) and *Spilostethus pandurus*.

variation in mate preference is associated with the diet of the focal individual. By comparing four separate species with similar ecologies we hope to identify more general effects of diet on patterns of variation in social behaviour.

2. Methods

2.1. Animal husbandry

L. equestris and its sister-species *L. simulans* are found across Northern and Central Europe where their preferred host is *Vincetoxicum hirundinaria*. *S. pandurus* is found in the tropics, sub-tropics and temperate zones of the old world while *O. fasciatus* is a new world species that ranges from the USA through to Central America and down into the Caribbean. The population of *L. equestris* we used in this study originates from bugs collected in the Dolomites mountains in northern Italy in 2004. The populations of *S. pandurus* and *L. simulans* were collected in Sicily in 2006. The *O. fasciatus* population was initiated from a long-term laboratory culture maintained by Dr Vernon French (University of Edinburgh). We maintained all four species in continuous culture in the laboratory subject to a fixed environmental regime of 22:2 h light:dark cycle and a temperature of 29 °C. These conditions prevented the bugs from entering reproductive diapause (Shuker et al., 2006). We used 30 cm × 15 cm × 15 cm plastic boxes with ventilated lids as stock cages, and provided organic, de-husked sunflower seeds (*Goodness Direct*, UK) at a depth of 3–4 cm across the bottom of the cage as food. We also provided two tubes of demineralised water with cotton wool bungs as a water source and changed these tubes weekly. Finally, cotton wool was also provided as a three-dimensional substrate for the bugs to sit on and hide in. In the lab all species are maintained on sunflower seeds, which appear to meet all their nutritional requirements. Stock populations are thus non-toxic. The common milkweed plant *Asclepias syriaca* is native to North

America, and thus *O. fasciatus* is the only species in this study that uses milkweed seeds in its natural diet. However, all species used in the study are able to sequester cardiac glycosides to some extent.

2.2. Experiment 1 – Nymph feeding preferences

Mated females of each species were isolated from stock cages (whilst in copula, with the male removed once mating complete) and housed individually in small plastic tubs containing sunflower seeds *ad libitum*, demineralised water and cotton wool for oviposition. Females were allowed to lay eggs which were left to hatch. Second instar offspring were collected and separated into two groups of approximately equal numbers, for each female. The two groups were then placed in 17 cm × 11 cm × 7 cm tubs containing either: (1) one (60 mm diameter) open plastic dish containing sunflower seeds (Dish S) and one plastic dish containing milkweed seeds (Dish M), subsequently referred to as the choice box; or (2) two plastic dishes containing sunflower seeds, referred to as the control box. Thus nymphs from a single mother were split between the two treatments. Water tubes were provided and regularly changed. Food was provided *ad libitum* across the developmental period. The mean number of nymphs per box was 19 at the beginning of the trial and 14 at the end (due to some nymphal mortality).

The boxes were surveyed each day between 10 and 11 am and the number of nymphs observed in each dish was recorded. This continued until the majority of the clutch reached 5th instar. This took approximately 11 days, but varied slightly between species. We also counted the total number of bugs surviving in each box every seven days. Nymphs from 56 females were included in the paired design resulting in a total of 112 boxes, half control and half choice for each species. The breakdown by species was as follows: 32 boxes contained *L. equestris*, 32 contained *L. simulans*, 20 *O. fasciatus* and 28 boxes contained *S. pandurus*. We tested for

differences among the four species in feeding preferences in two ways. First, we analysed feeding preferences using counts of the number of days nymphs were observed in each dish (nymphs observed in a food dish were assumed to be feeding) across the experiment (giving one count for each replicate). We used these counts to generate the proportion of days nymphs were observed feeding from one or both food dishes for each replicate (with number of days each replicate was surveyed as the denominator). We compared the proportion of days nymphs were observed feeding from sunflower and milkweed feeding dishes in the choice condition for the four species using a generalised linear model with binomial errors and a logit-link function (using IBM SPSS Statistics 19). Second, we compared the proportion of nymphs observed feeding from the S and M dishes in the choice condition for the four species using a generalised linear model with binomial errors and a logit-link function (again using SPSS). To again avoid pseudoreplication, each replicate provided only one data point representing the number of nymphs observed for each replicate divided by the total number of nymphs in the box. The average proportion across the surveys for each replication was then used as the data point for each replicate. Finally, we also checked the overall feeding rates in the presence of only sunflower or sunflower plus milkweed by comparing the feeding rates between choice and control boxes. We did this by comparing the proportion of nymphs observed in both dishes in both kinds of box (i.e. combining sunflower + sunflower for control boxes and sunflower + milkweed for choice boxes), again averaged across the experimental surveys for each replicate.

2.3. Experiment 2 – Nymph aggregation

Once the nymphs from experiment 1 reached 5th instar, ten nymphs from each treatment (where possible) were isolated and tested for aggregation behaviour. We set up 85 mm plastic Petri dishes on paper circles that were divided into four equal quadrants numbered 1–4, on the bench (24–25 °C). Nymphs were placed in an empty 30 ml Universal tube, which was then inverted over the centre of the Petri dish, thus introducing the nymphs into the centre of the arena together. At 30 and 60 min after introduction of the nymphs to the Petri dish we recorded the following: (1) how many nymphs were in each quadrant (if nymphs were aggregated together and over a section line we counted all nymphs in the aggregation as in the section that contained the majority of the group); (2) the number of aggregations in each dish (an aggregation was classed as two or more nymphs less than a body-length away from each other and not moving); (3) the number of nymphs in each aggregation; (4) the furthest distance between two nymphs in the dish. The effect of species, diet and their interaction on the number of nymphs in aggregations was analysed using a generalised linear model with a negative binomial (again in SPSS). Due to the non-normality of the data, the effect of species on the furthest distance between nymphs was tested using a Kruskal–Wallis test and the effect of diet on furthest distance was tested separately for each species with Mann–Whitney U tests (again in SPSS). We excluded dishes containing fewer than 10 nymphs, leaving us with a total of 50 trials, comprising: 18 *S. pandurus* replicates, 6 *O. fasciatus* replicates, 12 *L. equestris* replicates and 14 *L. simulans* replicates split between the two treatments.

2.4. Experiment 3 – Body lengths

Following mating trials (see below), bugs were frozen and stored at –20 °C. To test for any effects of diet on adult body size, we measured body length of all individuals after thawing using a dissecting microscope fitted with a measuring graticule. 614 individuals were measured: 188 *L. equestris*, 190 *L. simulans*, 50 *O. fasciatus* and 186 *S. pandurus*. We measured body length as the tip of the snout to



Fig. 2. The ventral view of a male *Lygaeus simulans*. The lines indicate the portion of the bug that was used for our measure of body length, i.e. from the tip of the snout to the end of the underside of the abdomen.

the end of the underside of the abdomen, with the bug lying on its back (Fig. 2). The effect of diet, sex and species on body length was tested using a general linear model with LSD post hoc tests to compare between the species.

2.5. Experiment 4 – Mating trials

Mating trials were performed over two weeks. Seven days after adult eclosion, males and females of the same species were paired up according to four combinations: (i) milkweed-fed male with milkweed-fed female; (ii) milkweed-fed male with sunflower-fed female; (iii) sunflower-fed male with milkweed-fed female; and (iv) sunflower-fed male with sunflower-fed female. Each individual was used in a single trial only. The pairs were introduced into plastic dishes 60 mm in diameter and observed continuously for a period of two hours, on the bench (24–25 °C). The order in which the two sexes were put into the dishes was randomised. We recorded the number of male mating attempts observed, as well as the initiation and duration of any copulations. In all four species, copulation is typically initiated by the male grasping the female with his legs and attempting genital coupling, with no obvious courtship phase. Pairs were classed as being in copula once they have moved into the end-to-end mating position typical of lygaeids (Micholitsch et al., 2000; Shuker et al., 2006).

The effect of treatment on mating success was analysed using logistic regression (i.e. a generalised linear model with a binomial error structure and logit-link function) in R 2.15.1, with male diet and female diet modelled as separate factors and species included as a main effect. A total of 318 pairs were included in this experiment: 36 *O. fasciatus*, 93 *S. pandurus*, 94 *L. equestris* and 95 *L. simulans*. Wald tests were used to test the significance of the main effects. The ratio of the residual deviance and the residual degrees of freedom was 1.2, so over-dispersion was not considered to be a problem.

3. Results

3.1. Experiment 1 – Nymph feeding preferences

We recorded two measures of feeding preference in the choice treatment: the average proportion of days bugs were observed feeding in either food dish, and the average proportion of nymphs observed in each food dish over all days. There was a significant preference for milkweed seeds over sunflower seeds in the

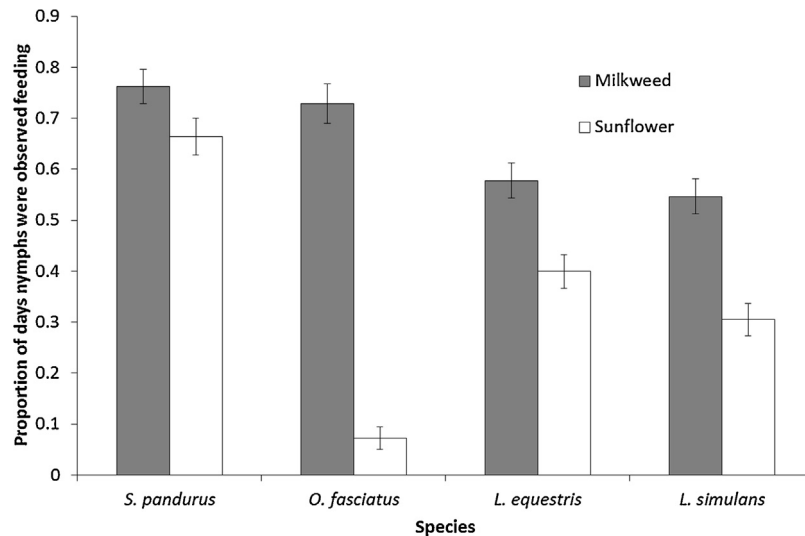


Fig. 3. Proportion of days that nymphs were observed in the different food dishes for each species in the choice (Sunflower & Milkweed) treatment. Bars indicate ± 1 standard error.

milkweed boxes, for both the proportion of days nymphs were observed in the different dishes (likelihood ratio test of the main effect of food type: $LR=209.36$, $d.f.=1$, $P<0.001$; Fig. 3) and the proportion of nymphs found in each dish (main effect of food type: $LR=5541.03$, $d.f.=1$, $P<0.001$; Fig. 4). However this pattern was not seen in all species: there was a significant species \times food type interaction, for both the presence in dishes ($LR=158.24$, $d.f.=3$, $P<0.001$) and the proportion of total nymphs in each dish ($LR=3368.28$, $d.f.=3$, $P<0.001$). *S. pandurus* nymphs did not show a significant preference for milkweed seeds while nymphs of the other three species did (Figs. 3 and 4). Moreover, *O. fasciatus* nymphs appeared to show the greatest preference for milkweed over sunflower seeds (Fig. 4). The four species also differed in their levels of feeding within the choice treatment, both in terms of the proportion of days nymphs were observed in both dishes (main effect of species: $LR=131.72$, $d.f.=3$, $P<0.001$) and in terms of

the proportion of nymphs observed in both dishes (main effect of species: $LR=14,587.35$, $d.f.=3$, $P<0.001$; Fig. 4).

Comparing across control (Sunflower only) and choice (Sunflower and Milkweed) treatments there were differences between the species in the overall level of feeding by nymphs ($LR=938.1$, $d.f.=3$, $P<0.001$): *S. pandurus* and *O. fasciatus* nymphs had a higher level of feeding than *L. equestris* and *L. simulans* nymphs ($P<0.001$ for all pairwise LSD contrasts) (Fig. 4). There was also a significant interaction effect between species and treatment ($LR=36.36$, $d.f.=3$, $P<0.001$), which is probably due to a higher level of feeding in the milkweed boxes by *S. pandurus*. Within the other three species the feeding rate was similar regardless of the presence of milkweed seeds, suggesting that overall nutritional requirements were being satisfied (or not satisfied) to the same extent in both treatments. Indeed, the main effect for treatment was not significant ($LR=0.44$, $d.f.=1$, $P=0.51$). The average proportion of nymphs

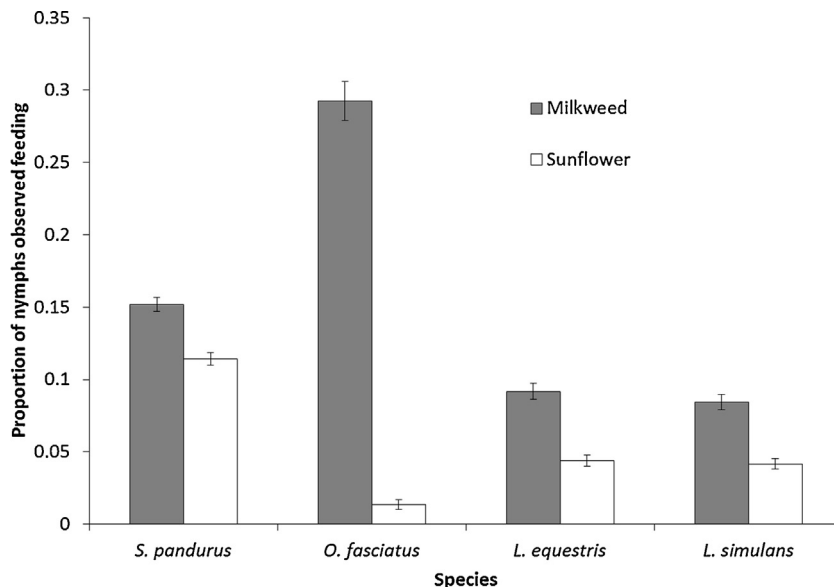


Fig. 4. Proportion of nymphs that were observed in the different food dishes for each species in the choice (Sunflower & Milkweed) treatment. Bars indicate ± 1 standard error.

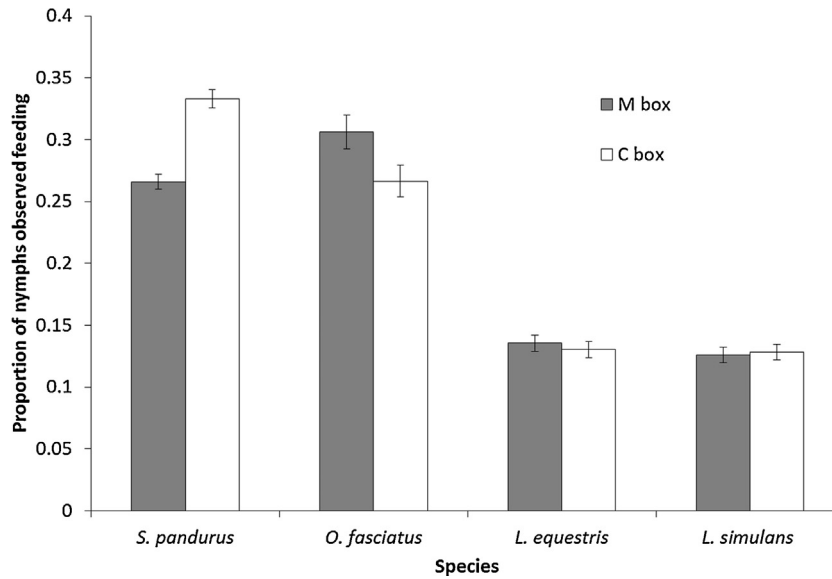


Fig. 5. Overall proportions of nymphs feeding in the milkweed (M) and control (C) boxes. Bars indicate the number of nymphs present in both dishes as a proportion of the total nymphs present in each box. Error bars indicate ± 1 standard error.

feeding in each dish for each treatment is displayed in Fig. 5. Our method of recording feeding preferences (counts once a day) means we are likely to have missed the majority of feeding events. Accordingly, the proportion of nymphs was never above 0.3 for any dish, across all species.

3.2. Experiment 2 – Nymph aggregation

Nymph aggregation behaviour after 30 or 60 min in dishes together did not differ between the species or in terms of diet ($P > 0.05$ in all cases including interactions). When we consider the furthest distance between two nymphs per dish however, some effects of species emerge. There were significant species differences in spacing after both 30 (result of Kruskal–Wallis test: $P = 0.04$, $d. f. = 3, 47$) and 60 min ($P = 0.007$, $d. f. = 3, 47$). The furthest distance between two nymphs in a dish was on average smaller in *O. fasciatus* and *S. pandurus* than in *L. equestris* and *L. simulans* (Fig. 6). Diet had no effect on the furthest distance between nymphs at either 30 or 60 min ($P > 0.05$ for all tests).

3.3. Experiment 3 – Body length

The four species used in the experiment differed significantly in body size (main effect of species: $F_{3,611} = 511.22$, $P < 0.001$). *O. fasciatus* was the smallest species with a mean body size of 10.7 mm, followed by *L. equestris* at 11.1 mm, then *L. simulans* at 11.9 mm. *S. pandurus* was the largest species with a mean body size of 12.8 mm. Females were significantly larger than males in all species (main effect of sex: $F_{1,613} = 677.85$, $P < 0.001$). The extent of sexual dimorphism varies between the species (interaction between species and sex: $F_{3,611} = 15.33$, $P < 0.001$) with *O. fasciatus* showing less sexual dimorphism than the other three species. The average body sizes of each species according to sex and species are displayed in Fig. 7.

Individuals fed on milkweed seeds were found to be significantly larger than those fed only on sunflower seeds (main effect of diet: $F_{1,613} = 18.29$, $P < 0.001$; Fig. 7). This is true in all cases except for *O. fasciatus* (Fig. 7). The effect of diet depended on sex as females showed a larger size increase when fed on milkweed than males (interaction between diet and sex: $F_{1,613} = 7.82$, $P = 0.005$).

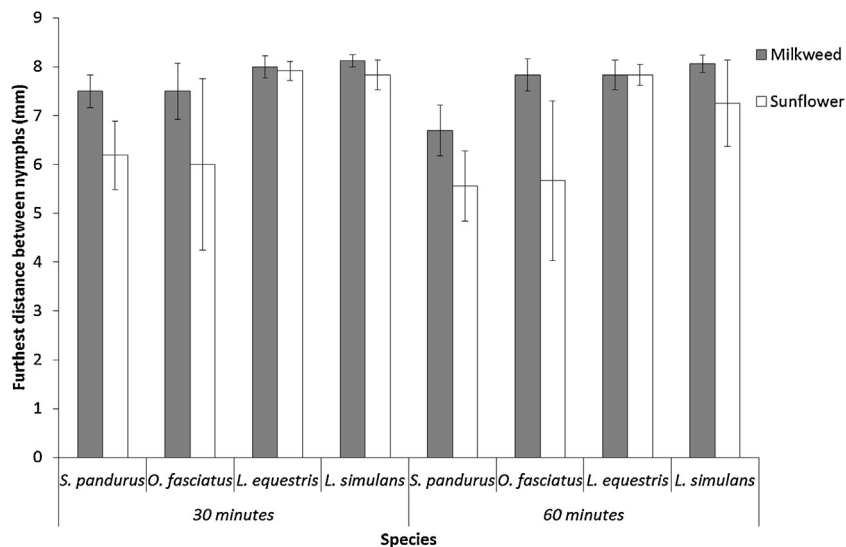


Fig. 6. The mean furthest distance between two nymphs at 30 and 60 min (when dishes with fewer than 10 nymphs were excluded), bars indicate ± 1 standard error.

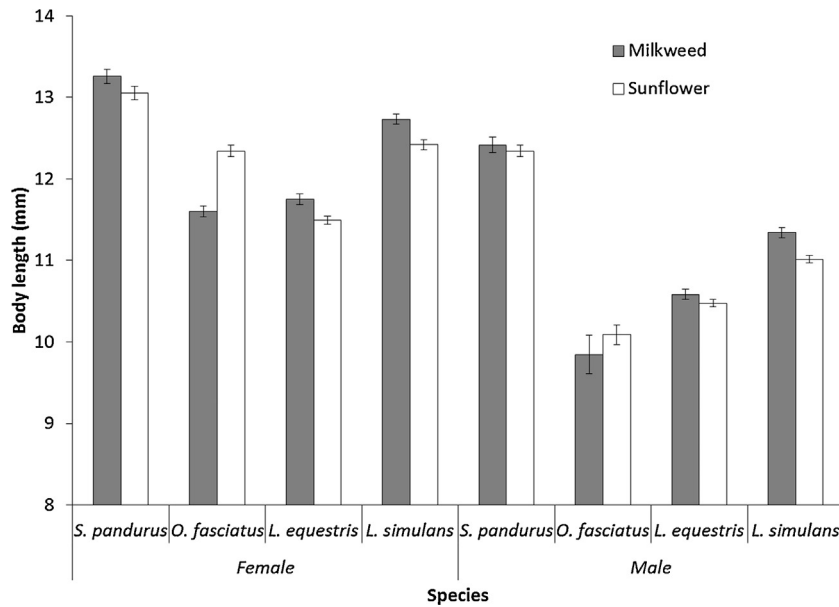


Fig. 7. The mean body length of each species for females and males according to diet and species. Bars indicate ± 1 standard error.

However, diet did not change overall patterns of size variation among the four species (interaction between diet and species: $F_{3,611} = 1.407, P = 0.24$), nor did the interaction between species, diet and sex significantly affect body size ($F_{3,611} = 2.02, P = 0.109$).

3.4. Experiment 4 – Mating trials

Across all the trials, approximately 57% of pairs copulated during the 2 h observation period. Mating frequency varied between species, independently of diet ($\chi^2 = 39.88, df = 3, P < 0.001$). *L. equestris* and *O. fasciatus* both had higher rates of mating (75% and 81% of pairs respectively) than *L. simulans* and *S. pandurus* (48% and 38% of pairs respectively) (Fig. 8). Contrary to our predictions, milkweed-fed males had significantly lower mating success than sunflower-fed males ($\chi^2 = 7.90, df = 1, P = 0.005$). This pattern was most strongly influenced by the decreased mating frequency observed in *L. equestris* males when fed on milkweed and the effect

becomes non-significant when *L. equestris* is removed from the analysis ($\chi^2 = 1.60, df = 1, P = 0.205$). There was no difference in mating success for females fed either milkweed or sunflower seeds ($\chi^2 = 2.07, df = 1, P = 0.15$). There were also no significant interactions between any of the factors ($P > 0.05$ in all cases), such that the attractiveness of one sex was not influenced by the diet of the other.

4. Discussion

We tested the feeding preferences and aggregation behaviour of nymphs of four species of aposematic Lygaeidae, as well as their adult mating preferences, to look at the effect of early diet on social behaviour in these insects. We found only a limited effect of diet on key social interactions. Contrary to our predictions, our results showed no influence of diet on the aggregation behaviour of nymphs. While the species clearly differed considerably in their propensity to aggregate, no significant effect of diet on aggregation

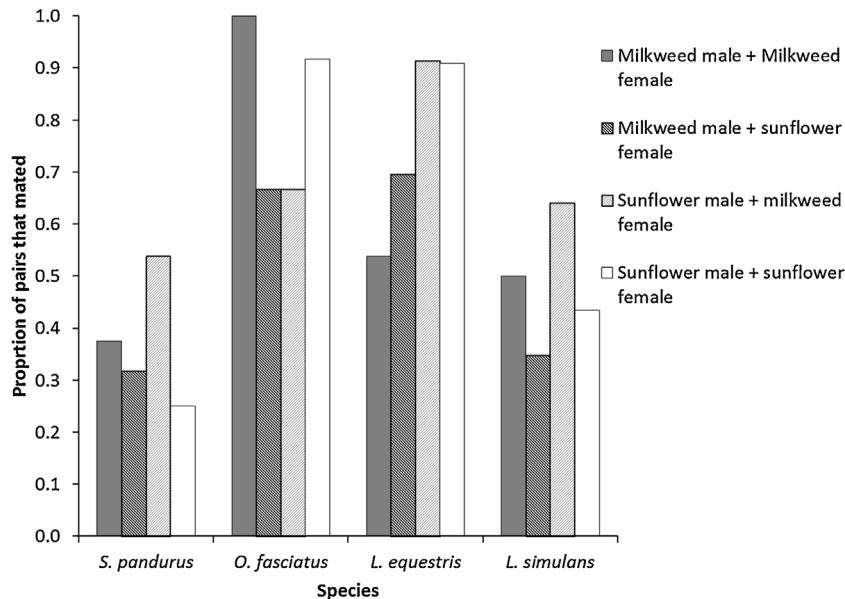


Fig. 8. The proportion of pairs which mated during the course of the trial for each species and treatment.

behaviour was found. *O. fasciatus* and *S. pandurus* both readily formed aggregations regardless of diet while *L. equestris* and *L. simulans* were less gregarious in nature. The effect of diet on mating behaviour also ran contrary to our predictions. Despite both sexes showing larger body sizes when fed milkweed, only in one species did diet affect mating success: in *L. equestris*, milkweed-fed males had lower mating success than those fed on sunflower seeds. Diet had no effect on female mating success in all species tested.

While aggregation has been shown to increase the protection associated with aposematic signals (Tullberg et al., 2000), it may be that aggregation in these species is not state-dependent but rather an evolved response regardless of the level of individual chemical protection. As even nymphs living in close proximity may vary in their chemical defence due to food availability, it could be the case that a relatively unprotected individual may be surrounded by well-protected conspecifics and thus still benefit from the increased aposematic signal presented by aggregation. Research has shown that predators with experience of protected prey items will avoid other individuals of that species regardless of their level of chemical protection (Berenbaum and Miliczky, 1984). For example, praying mantids with experience of milkweed-fed *O. fasciatus* adults would then avoid bugs fed on sunflower seeds, despite these being palatable to naive mantids (Berenbaum and Miliczky, 1984). If group size increases the level of protection from predators for all members of the group then it may be advantageous for protected nymphs to associate with any conspecifics in the area, regardless of their level of protection.

In terms of the life-history consequences of nymph diet, we found that for all species (except *O. fasciatus*) adult individuals were larger when fed on milkweed seeds. The lack of a response in *O. fasciatus* is more puzzling as milkweed is its natural food plant and thus we might expect to see greater increases in body size of milkweed-fed individuals. A recent study has suggested that milkweed-fed *O. fasciatus* males invest more of their resources in mating and reproduction at the expense of longevity (Attisano et al., 2012), although this study only manipulated adult diet. Previous evidence suggests that adult body size is strongly affected by nutrition during development (Blakley and Goodner, 1978; Solbreck et al., 1989; Blakley, 1981a, 1981b), especially in 5th instar nymphs, suggesting dietary effects may affect nymphs and adults differently (Blakley, 1981b). Attisano and colleagues suggested that the differences in resource allocation between the two diets in their study were the result of a shift in life-history trade-offs that accompanied the adaptation of *O. fasciatus* to sunflower seeds. Adaptation of most seed bug species to sunflower seeds takes place very quickly in a laboratory environment, and all the populations used in this experiment have been raised on sunflower seeds for many generations prior to their use in this experiment. Thus the lack of change in body size in *O. fasciatus* may be reflective of different life history trade-offs operating in this species versus *S. pandurus*, *L. equestris* and *L. simulans*. However, we must be cautious in interpreting this finding as it may be an artefact of the smaller sample size for *O. fasciatus* compared with the other species.

No increase in mating success was detected in the milkweed-fed individuals, as would be expected if sequestered chemical protection increased attractiveness to the opposite sex. Indeed while male diet did appear to affect the rate of successful mating it was in the opposite direction to that predicted. This was especially pronounced in *L. equestris* where males fed only on sunflower seeds had a much higher mating rate than those fed on milkweed seeds. However, it must be noted that sunflower-fed males only enjoyed greater mating success in *L. equestris*. If this species is removed from the analysis the effect of male diet on mating success becomes non-significant. It could be that the variation in effects observed across the species is not caused by variation in the potential levels of chemical defence but by some other aspect of nutrition.

The reason for this is unclear however; it may be related to the finding that milkweed-fed males had a greater body size as there is suggestion that female *L. equestris* prefer smaller males (Dougherty & Shuker, unpublished results). In contrast, we might expect increased body size to correlate with improved nutrition and it has been suggested that in other organisms females may prefer well-fed males (Giaquinto et al., 2010). Milkweed is not the natural diet of *L. equestris* so it could be that the effect observed in this species is caused not by variation in levels of chemical defence but by some other aspect of nutrition. For example, improved nutrition has been found to correlate with increased courtship behaviour and mating success in male wolf spiders (*Pardosa prativaga*) (Lomborg and Toft, 2009) and increased reproductive success in spruce budworms (*Choristoneura fumiferana*) (Delisle and Hardy, 1997). The lack of a significant male preference for females fed on milkweed (and thus assumed to possess greater levels of cardiac glycosides) is also unexpected as very recent work has found that female *O. fasciatus* may pass defensive chemicals to their eggs, thus giving eggs some protection from predators (Newcombe 2013 unpublished thesis).

Mate choice outcomes were tested using a no-choice paradigm, in which each bug was tested once with a single potential mate. No-choice paradigms can test for absolute mating preferences, but are probably less likely to detect small differences in preference between stimuli (Wagner, 1998; MacLaren and Rowland, 2006). When compared to choice tests, rejection of a mate in a no-choice test may be more costly due to the potential risk of lost mating opportunities (Barry and Kokko, 2010; Booksmythe et al., 2011). However, we have recently shown little variation in mating outcomes between choice and no-choice paradigms in *L. equestris*, based on male and female morphology (Dougherty & Shuker, in prep), so perhaps this criticism is less relevant here. What perhaps is more important is that Dougherty and colleagues showed rather little pre-copulatory choice in *L. equestris*, across all experimental paradigms. To date, studies of pre-copulatory sexual selection in lygaeids have shown some patterns of non-random mating in terms of aspects of morphology in both males and females (Yang and Wang, 2004; McLain, 1992). While mate choice may occur either before, during or after copulation, our experiment only tested pre-copulatory preferences. There is growing evidence that post-copulatory sexual selection may play a greater role in lygaeids, as indicated by the extreme genital morphology in these species (Gschwentner and Tadler, 2000; Micholitsch et al., 2000; Bonhag and Wick, 1953), and evidence for post-copulatory selection on aspects of male and female genitalia (Tadler et al., 1999; Yang and Wang, 2004; Higgins et al., 2009). All four species exhibit very long copulations (Walker, 1979; Sillen-Tullberg, 1981; Micholitsch et al., 2000), which likely function as a form of post-copulatory mate-guarding (Tadler et al., 1999; Sillen-Tullberg, 1981), as well as a high frequency of mating failures, even after successful intromission by the male (Tadler et al., 1999; Micholitsch et al., 2000). This suggests that sperm competition and potentially cryptic female choice may play a significant role in sexual selection in these species. Future experiments that test cryptic female choice for males in terms of nutrition or chemical defence may be more fruitful (see also Newcombe, 2013 unpublished thesis).

Despite the apparent lack of strong effects on behaviour, *Ls equestris*, *L. simulans* and *O. fasciatus* nymphs all showed a preference for feeding on milkweed seeds over sunflower seeds when provided with both. The only species in this trial not to show this preference was *S. pandurus*. However, *S. pandurus* was observed in the food dishes more frequently than any of the other species and this may have masked any weaker diet preferences. It is also possible that as *S. pandurus* is one of the larger species used in this experiment (see experiment 4) there may have been greater competition for food within the *S. pandurus* boxes.

While milkweed was clearly preferred as a food source by the nymphs of all but one of the species using in this study, diet did not play a significant role in determining the social behaviour of the bugs. While there was variation among the different species, neither the propensity to aggregate as nymphs, nor mate choice as adults, was much associated with diet. While our study did not measure chemical defence directly, these findings suggest that social behaviour in the Lygaeidae may depend less on nutritional or chemical-defence state than previously thought. Rather than adjusting their behaviour in response to either their own internal states, or that of others, lygaeids may instead base their social interactions on behavioural “rules of thumb” which are advantageous in the majority of circumstances. The next step will be to explore patterns of social behaviour in terms of nutrition and chemical defence in the wild. Our data here suggest that processes like automimicry, where individuals vary in their chemical defence, and perhaps are completely undefended yet still perform aggregation behaviour, may be more common (Speed et al., 2012).

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