Comparative phenotyping across a social transition in aphids

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In some insects, eusociality has evolved independently more than once, such that closely related species differ in the presence or absence of altruistic traits. Such groups offer opportunities to study the ecological and evolutionary drivers of transitions to sociality. In *Pemphigus* aphids, for example, eusociality has evolved independently multiple times, but most species are assumed to be nonsocial. Eusocial aphids thus typically have close relatives that are nonsocial, indicating a rapid and distinct transition to sociality. However, there has been only limited study of the behaviour of nonsocial species that permit direct comparisons with eusocial species. In this study, we characterized three aphid species along two axes of social behaviour: housekeeping and defence. Previous evidence suggested that these three species differ in the presence or absence of social traits. We found that for the ecological and behavioural traits we tested, there were quantifiable differences between social and nonsocial species. However, there was no clear threshold that differentiated social from nonsocial species, meaning that definitions of sociality in aphids depend in part on the traits that are measured. If sociality is measured by defence, for example, the eusocial species, *Pemphigus obesinymphae* clearly expressed the greatest degree of aggressive and effective defence. However, some defensive behaviour was also present in the species traditionally defined as nonsocial. Conversely, if sociality in aphids is measured by traits related to homeostasis and housekeeping, then the species traditionally considered nonsocial expressed nearly the same behaviours as the eusocial species. These results imply that sociality in aphids evolves as a collection of uncorrelated traits. Clear analogues or antecedents of more derived social characters can be identified in species that are nominally nonsocial.

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One of the major transitions in animal evolution is the shift from solitary to social lifestyles (Queller, 2000; Szathmary & Smith, 1995). The goal of comparative studies of social evolution is to understand the factors involved in this transition and to answer questions such as (1) what are the commonalities of social behaviour across socially living taxa and (2) what are the ecological/demographic/life history predictors of these commonalities (Bourke, 1999; Danforth, 2002; Ratnieks, Foster, & Wenseleers, 2006; Ross, 2001)? Such questions are addressed with phylogenetic comparative approaches and ideally, detailed ecological analyses of related nonsocial and social species. However, ecological studies that bridge social transitions are often not possible in many species with advanced eusociality either because nonsocial species are absent, or because the evolutionary change between taxa is so great that the interpretation of ecological features is difficult. Consequently, species that express sociality facultatively or in

which sociality is phylogenetically labile are valuable in comparative studies of social evolution (Gunnels, Dubrovskiy, & Avalos, 2008; Soro, Field, Bridge, Cardinal, & Paxton, 2010; Wcislo, 1997). Social aphids offer unique opportunities for comparative studies, because sociality exhibits a remarkable degree of evolutionary lability and has been independently gained and lost multiple times among closely related species (Abbot, 2009; Pike, Whitfield, & Foster, 2007; Stern, 1994).

Aphids are small, soft-bodied herbivorous hemipterans that feed exclusively on plant phloem. There are about 5000 species, and many have complex life cycles that span two or more host plants and that alternate between sexual and asexual generations. Sociality is rare in aphids, and nearly all social species are found in two subfamilies in the Aphididae: Hormaphinae and Eriosomatinae (Stern & Foster, 1996). Unlike better-studied eusocial taxa, such as bees and ants, social aphids do not express cooperative brood care. Rather, the defining feature of aphid sociality is aggressive, self-sacrificial defence against natural enemies by wingless subadult females, often involving specialized morphology or other weaponry (Stern & Foster, 1996, 1997). These females are 'soldiers', and what they defend is their kin groups, which aggregate in dense

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clusters of clonally produced females often within tumour-like plant growths known as galls. Gall-forming has evolved independently several times in aphids, and while the complexity of aphid life cycles makes it difficult to generalize about what exactly causes groups to form (in some species, soldiers are present during non-galling points in the life cycle), at the species level, sociality has rarely been observed in species that do not form galls at some point. Because of fierce defence of the nest-like gall by nymphal soldiers, a habit they share with other species that defend nests and refuges, such as termites, thrips, snapping shrimp and Damaraland mole-rats, *Cryptomys damarensis*, aphids are considered 'fortress defenders' (Cooney, 2002; Crespi, Carmean, & Chapman, 1997; Duffy, 1996; Queller & Strassmann, 1998; Sobotnik, Jirosova, & Hanus, 2010). Note that this is somewhat overly simplified when it comes to aphids, because of the presence of soldiers in some species when no gall or 'fortress' is present.

One of the most valuable contributions of primitively eusocial insects like aphids is the potential they offer for comparative studies of social evolution, because closely related species, often sharing the similar habitats or overlapping distributions, seem to differ in the presence or absence of sociality. However, an unresolved issue is that what precisely constitutes a social aphid species is not obvious, limiting the practical use of comparative studies of social and nonsocial species. In some species, the presence of morphologically specialized or even reproductively sterile soldiers is unmistakable. But in others, the threshold for sociality has traditionally been defined by a combination of life history traits and behaviours. Most species have not been formally examined at all. Pike and Foster (2008) pointed out that many more aphid species live in groups than are nominally defined as social, and in those, social behaviours may be cryptically expressed, suggesting unappreciated complexity in aphid sociality. Moreover, aphids can express a range of behaviours that some authors describe as social. The primary social behaviour not related to defence in aphids is 'housekeeping', in which some group members actively remove waste and cadavers to prevent fouling of the gall, in a manner similar to that of other eusocial insects (Sun & Zhou, 2013). According to Benton and Foster (1992), this behaviour in aphids is likely an act of kin-selected altruism, because the energetically expensive or even dangerous act of cleaning confers a group-level benefit. The presence of housekeeping behaviours may indicate rudimentary forms of cooperative group living in aphids that, from a comparative perspective, represent precursors or evolutionary routes to more advanced sociality in aphids. No study has yet attempted to study evolutionary correlations between defence and other social behaviours in aphids in a phylogenetic framework. It is not known whether, across different aphid species, sociality is expressed as syndromes of covarying cooperative behaviours, or whether these behaviours vary independently, with species that express various combinations of social traits. In short, it is unclear what a social transition is in aphids.

What, then, is sociality in aphids, and how is it measured? Most studies of social behaviour in aphids have focused on single species. There have been relatively few comparative studies of social aphid evolution (Shingleton & Foster, 2001; Stern, 1994) or that have placed single specie studies into comparative frameworks (Shibao, Shimada, & Fukatsu, 2010), and only one that has systematically evaluated the behaviours of congeneric aphids that vary in the expression or degree of social behaviour (Rhoden & Foster, 2002). In this study, we evaluated two axes of social behaviour in aphids (housekeeping and defence) in a group of congeneric North American aphid species. The three closely related species we studied each form galls on *Populus* spp. but vary in the degree to which they express social defence behaviours. Two species, *Pemphigus populitransversus* and *Pemphigus obesinymphae* are sister

species, while the third species, *Pemphigus populicaulis* is more distantly related in a monophyletic group that includes all North American *Pemphigus* species (Abbot & Withgott, 2004). Our goal was to define objectively a social transition in aphids. Below, we compare the ecology and life history of these three species. Next, we use behavioural assays to compare another feature of sociality in aphids, altruistic housekeeping. Finally, we use an objective measure of aphid sociality, natural predator morbidity, to provide a common scale on which to rank the species in terms of social behaviours. Our results indicate that there is no evidence of sharply defined social thresholds in *Pemphigus* aphids, much as Pike and Foster predicted (2008), and the foundations of the most derived social behaviours can be gleaned in species that are not traditionally defined as social. However, our results also indicate that clear and objective demarcations of social behaviour in aphids can be identified. The most unambiguous of these is the ability to kill much larger insects than themselves.

METHODS

Some background on the basic biology of *Pemphigus* aphids is helpful in understanding their social biology. Aphids are hemimetabolous insects, meaning that unlike ants and bees, which are holometabolous, they do not undergo complete metamorphosis from juveniles to adults. Rather, the juveniles are typically morphologically similar to adults and undergo a serious of moults from an initial or first instar, through progressively larger instars before reaching adulthood. Thus, whereas holometabolous social insects have relatively immobile larva that may be provisioned by workers, aphids and their allies have mobile larvae that are capable of feeding themselves. In *Pemphigus*, social behaviour occurs within galls and is expressed primarily by the first-born (first instars). Aphids express viviparous parthenogenesis, which means that an 'army' of clonal nymphal soldiers can rapidly accrue during colony development.

Ecology of Pemphigus populicaulis: an Aphid without Social Behaviour

Pemphigus is a genus of aphids in the holarctic subfamily Eriosomatinae (Aphidoidea: Aphididae; formerly Pemphiginae). *Pemphigus populicaulis* (Fitch) is a gall-forming species that is widely distributed across North America (Blackman & Eastop, 1994). This species has a typical heteroecious life cycle, involving annual alteration between primary hosts in the genus *Populus* (primarily *Populus deltoides* or *Populus tremuloides*), on which sexual generation and gall formation occur, and an undetermined secondary host plant (Blackman & Eastop, 1994). Galls are initiated in the spring on the petiole at the base of the leaf blade. Within the gall, and as with the species described below, the foundress reproduces parthenogenetically. Four wingless instars occur in rapid succession before a final moult into winged alates, which migrate in late spring (April—May) to the secondary hosts, ultimately returning to poplars, where a sexually produced, overwintering egg is laid beneath the bark (Table 1). With only a brief galling phase on poplars, *P. populicaulis* does not appear to express defence and, thus, has been described as 'nonsocial' (Abbot, 2009).

Ecology of Pemphigus populitransversus: an Aphid with Intermediate Social Behaviour

Pemphigus populitransversus Riley distribution is limited to the eastern United States (Blackman & Eastop, 1994). Although closely related to *P. obesinymphae* (see below), the life cycle of this species is similar to the life cycle of *P. populicaulis*. *Pemphigus populitransversus* has been reported on many *Populus* spp., but most

Table 1
Ecology of the three aphid species examined

Trait	*Pemphigus populicaulis*	*Pemphigus populitransversus*	*Pemphigus obesinymphae*
Gall			
Distribution	North America	Eastern U.S.A.	U.S.A. and northern Mexico
Primary host plant (*Populus* spp.)	*P. deltoides* and *P. tremuloides*	*P. deltoides* and *Populus* spp.	*P. deltoides* and *P. fremontii*
Secondary host plant	Compositae	Brassicaceae	Brassicaceae
Location of gall	On petiole near leaf lamina	Petiole	On petiole near leaf lamina
Month of gall induction	March	April	May
Gall length	2–3 months	3–4 months	5–6 months

frequently forms galls on *P. deltoides*, alternating between these and a secondary host in the Brassicaceae (Table 1; Blackman & Eastop, 1994). Galls are initiated later in the spring than are those of *P. populicaulis* (Abbot & Withgott, 2004). Both species, however, form galls on the petioles of the first or spring flush of leaves that sprout from the pre-formed overwintering poplar buds. Previous work has suggested that first-instar nymphs express some degree of defensive behaviours and, thus, *P. populitransversus* is 'weakly social' and represents a transitional phase in social behaviour in aphids (Pike et al., 2007; Rhoden & Foster, 2002).

Ecology of Pemphigus obesinymphae: *an Aphid with Highly Social Behaviour*

Pemphigus obesinymphae is distributed across the United States and into northern Mexico (Blackman & Eastop, 1994). Although closely related to *P. populitransversus*, the life cycle of *P. obesinymphae* is unusual, differing from the traditional life cycle of many *Pemphigus* aphids (Abbot & Withgott, 2004). Its life cycle incorporates a different overwintering strategy in which it has omitted diapause all together. The life cycle is temporally rotated forward, overwintering on the secondary plant (Brassiceae), rather than returning to the primary host (*Populus fremontii* or *P. deltoides*) in the autumn. Unlike *Pemphigus populicaulis* or *P. populitransversus*, the galls of *P. obesinymphae* are initiated on the second or summer flush of leaves, which sprout from newly formed buds (Table 1). *Pemphigus obesinymphae* has been described as bearing the hallmarks of eusociality in aphids, including aggressive behaviours by nymphs, which either delay or do not develop adults, and an unusually prolonged galling phase (Moran, 1993; Stern & Foster, 1996).

Altruistic Housekeeping

In all of the social *Pemphigus* species, it is the first instars that express defensive behaviours. Likewise, housekeeping by first-instar nymphs has been demonstrated in two other *Pemphigus* species, *Pemphigus spyrothecae* and *Pemphigus dorocola* (Aoki, 1980; Benton & Foster, 1992). Housekeeping can be characterized in gall-forming aphids by observing the active removal of waste from the galls, or even more dramatically, active and altruistic repair of galls that have been damaged (Kutsukake, Shibao, Uematsu, & Fukatsu, 2009). Many aphid species produce copious amounts of powdery wax, and it is thought that wax assists the aphids in managing the large amount of honeydew fluids that they excrete, either by coating the honeydew into manageable packages ('balls') that can be removed from galls more efficiently, or perhaps, by reducing the likelihood of growth by opportunistic microbes on the sugary honeydew (Pike, Richard, Foster, & Mahadevan, 2002; Pope, 1983; Smith, 1999). Many species form galls that do not have openings to the outside, but in those that do, waste can simply fall out due to gravity. By manipulating the position of the gall opening, or ostiole, and thus preventing removal by gravity, active housecleaning can be measured. In nature, for unclear reasons, the ostiole is often not angled down, but is often at various, oblique angles. In *P. spyrothecae*, the typical angle is about 30° from a line drawn perpendicular to the ground and the gall (Benton & Foster, 1992). To examine whether first instars express housekeeping behaviours in other *Pemphigus* species, we removed first instars from the gall and scored the effect on waste removal. Galls of all three species were
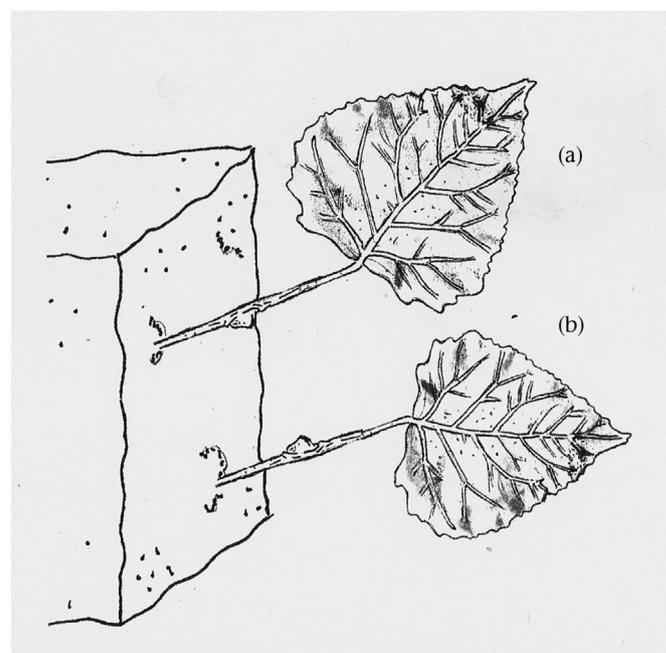
Figure 1. Illustration of a *Pemphigus populitransversus* gall in floral foam with (a) the ostiole in its natural downward direction and (b) the ostiole oriented upwards.

collected from sites around Dyersburg and Nashville, TN, U.S.A., in the summer of 2012 and 2013. In the laboratory, a small piece of the gall was removed and the gall was emptied of aphids. Into these galls, we either (1) replaced 100 aphids (50 first instars and 50 late instars, $N = 10$ galls), or (2) replaced 90 aphids (30 second instars and 60 late instars, $N = 10$). The remaining aphids were immediately placed into the freezer for long-term storage. The goal was to control for the biomass of the aphids and the varying rates of honeydew production (Benton & Foster, 1992). To retain the turgor pressure of the leaves, the petiole of the leaf containing the gall was placed in floral foam with the ostiole in its natural orientation (Fig. 1a). Petri dishes were placed under each gall and the contents of the dishes tallied and removed daily for 7 days. At the end of experiment, galls were taken down and the contents tallied. The total number of aphids, number of ejected exuviae (aphid exoskeletons) and honeydew balls (aphid waste) were analysed as nonparametric equivalents of two-way ANOVAs. Whole model tests were followed by pairwise contrasts (Sokal & Rohlf, 1995). All statistical analyses were performed in JMP v. 7.01 (SAS Institute, Cary, NC, U.S.A.). All reported P values are two tailed.

To measure the amount of honeydew production of each species, we manipulated the direction of the ostiole. Galls of three Pemphigus species were collected from multiple sites around Nashville, TN during the spring and summer of 2013. For each species, we placed two leaves with galls in floral foam, one with the ostiole pointing in its natural direction ($N = 12$; Fig. 1a) and one with the ostiole pointing directly up ($N = 12$; Fig. 1b), to measure the number of honeydew balls and exuviae actively pushed from galls in each position. The floral foam was placed in water to prevent the galls from desiccating during the duration of the experiment. After 5 days, we removed the galls and tallied the total number of aphids and honeydew balls remaining in the gall.

Fortress Defence

We collected galls of the three Pemphigus species, P. populicaulis, P. populitransversus and P. obesinymphae, from sites in the Nashville, TN area between March and August 2013. Galls were collected and immediately used in behavioural assays. We placed a predator or Drosophila melanogaster larvae into the gall through the ostiole. We used two known predators of Pemphigus aphids, including green lacewing larvae (Neuroptera, Chrysopidae, Chrysoperla rufilabris) and minute pirate bugs (Hemiptera, Anthocoridae, Orius spp.) (Wilch, 1999). Previous studies have used third-instar D. melanogaster (Diptera, Drosophilidae) to mimic dipteran larval predators (Abbot, Withgott, & Moran, 2001). We monitored the predator or D. melanogaster larvae every 20 min and noted survivorship. Controls involved placing these insects in empty galls without aphids to establish each predator's baseline survival. The interior surfaces of Pemphigus aphid galls are covered in a waxy substance that the aphids exude and which likely acts to deter predators (Pope, 1983). Experiments with Orius spp. ended after 8 h and those with C. rufilabris ended after 12 h, because the Orius spp. were only tested against P. obesinymphae soldiers. Orius spp. were only tested against P. obesinymphae soldiers because they are frequently found in galls of P. obesinymphae but not as frequently found in the galls of the other species (S. P. Lawson & P. Abbot, personal observation). The sample size for each group was at least 20 galls. We recorded survival of D. melanogaster larvae every 20 min and performed statistical analyses using the log-rank survival test. Differences were considered significant at $P < 0.05$. To further characterize defence, we introduced third-instar D. melanogaster larvae to the gall and counted the number of aphids actively attacking the larvae every 15 min for 180 min ($N = 20$). We compared the average number of attackers for each

species using a Wilcoxon Lifetest. All statistical analyses were performed in JMP v. 7.01 (SAS Institute).

Ethical Note

All aphids and predators were placed in a freezer at the conclusion of the experiment. Fortress defence experiments were similar to interactions observed in nature. Unfortunately, there is no better measure of soldier effectiveness than using a natural or pseudopredator.

RESULTS

### Altruistic Housekeeping Behaviours

Housekeeping behaviour was observed in the putatively nonsocial P. populicaulis as well as in the weakly social and eusocial
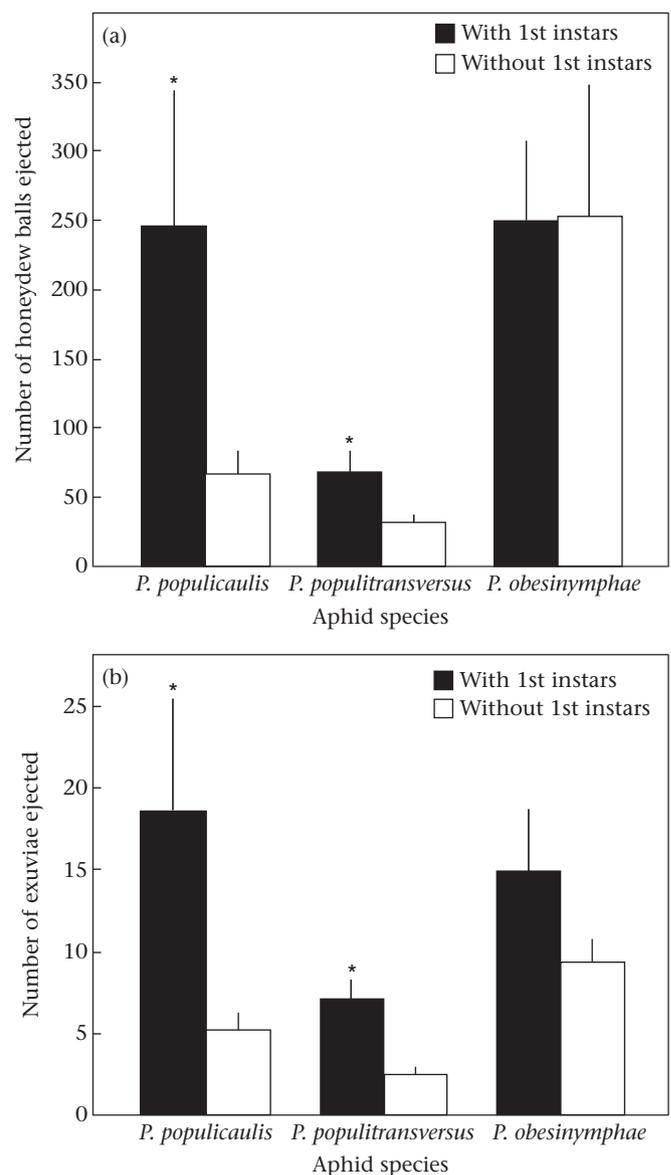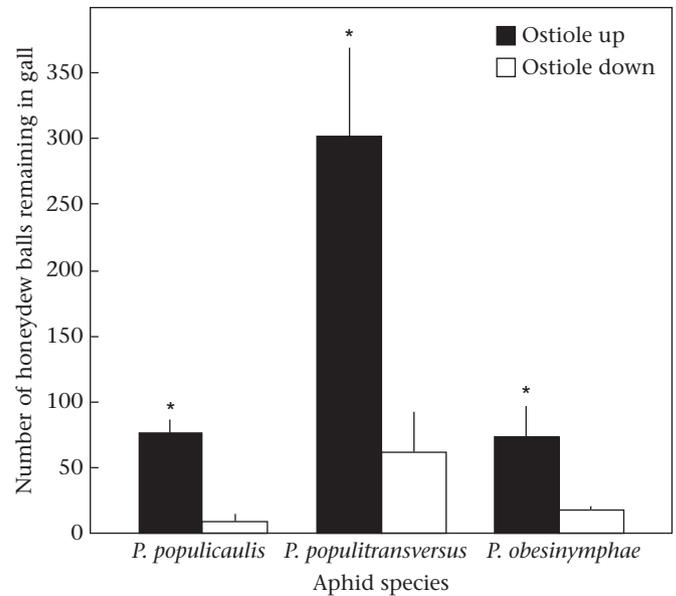

Figure 2. Average ± SE number of (a) honeydew waste balls and (b) aphid exoskeletons ('exuviae') ejected from the gall of three aphid species. Each gall contained 100 aphids (50 first instars, 50 late instars; $N = 10$ galls; black bars) or 90 aphids (30 second instars, 60 late instars; $N = 10$; white bars). Asterisks indicate a significant difference at ($P < 0.05$).

species. However, the individuals that performed this behaviour differed between species. In both *P. populicaulis* and *P. populitransversus*, housekeeping was mostly performed by first-instar nymphs, whereas in the eusocial species, all larvae performed housekeeping duties. Groups with first instars ejected more honeydew balls over the 7-day period than groups without first instars (Wilcoxon Lifetest: *P. populicaulis*: $\chi^2_1 = 6.7583$, $P = 0.0093$; *P. populitransversus*: $\chi^2_1 = 8.6628$, $P = 0.0032$; Fig. 2a). Surprisingly, there was no significant difference in the number of honeydew balls ejected with or without first instars in *P. obesinymphae* galls (ANOVA log transformation: $F_{1,16} = 1.6038$, $P = 0.2247$; Fig. 2a). Overall, more honeydew balls were ejected from groups of eusocial *P. obesinymphae* and nonsocial *P. populicaulis* than from weakly social *P. populitransversus* groups (ANOVA log transformation: $F_{5,47} = 13.3842$, $P < 0.0001$; Fig. 2a, Supplementary Video S1). Similar trends were seen in the ejection of exuviae from the gall over the 7-day period. Groups with first instars ejected more exuviae over the 7-day period than groups without first instars in both *P. populicaulis* (Wilcoxon Lifetest: $\chi^2_1 = 6.0814$, $P = 0.0137$) and *P. populitransversus* ($\chi^2_1 = 5.3343$, $P = 0.0209$), while in *P. obesinymphae* groups, the presence or absence of first instars made no difference in the amount of exuviae ejected (ANOVA with log transformation: $F_{1,16} = 0.5773$, $P = 0.4584$; Fig. 2b). We found no significant difference between the number of winged alates or the number of dead aphids in galls with and without soldiers for any species (Supplementary Table S1).

There was a significant difference in the number of aphids in the gall of each species (Wilcoxon Lifetest: $\chi^2_2 = 86.7542$, $P < 0.0001$; *P. populicaulis* versus *P. populitransversus*: $P = 0.4191$; *P. populicaulis* versus *P. obesinymphae*: $P < 0.0001$; *P. populitransversus* versus *P. obesinymphae*: $P < 0.0001$; Fig. 3). Because aphid densities within galls varied across species, we normalized the number of honeydew balls ejected against the average number of aphids per gall for that species. First, changing the normal position of the ostiole (from angled towards the ground to pointing directly upward) clearly reduced the ability of each aphid species to eject waste (Wilcoxon Lifetest: *P. populicaulis*: $\chi^2_1 = 21.5628$, $P < 0.0001$; *P. populitransversus*: $\chi^2_1 = 10.8609$, $P < 0.001$; *P. obesinymphae*: $\chi^2_1 = 12.5374$, $P = 0.0005$; Fig. 4). Second, comparing across species, *P. populitransversus*, the species with weakly expressed
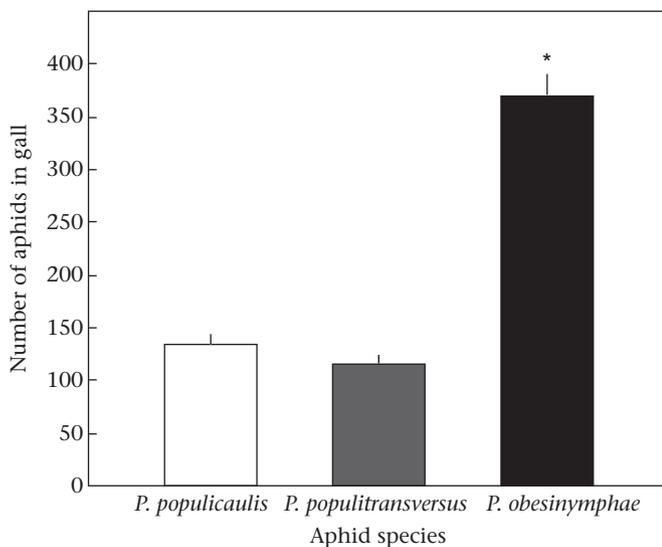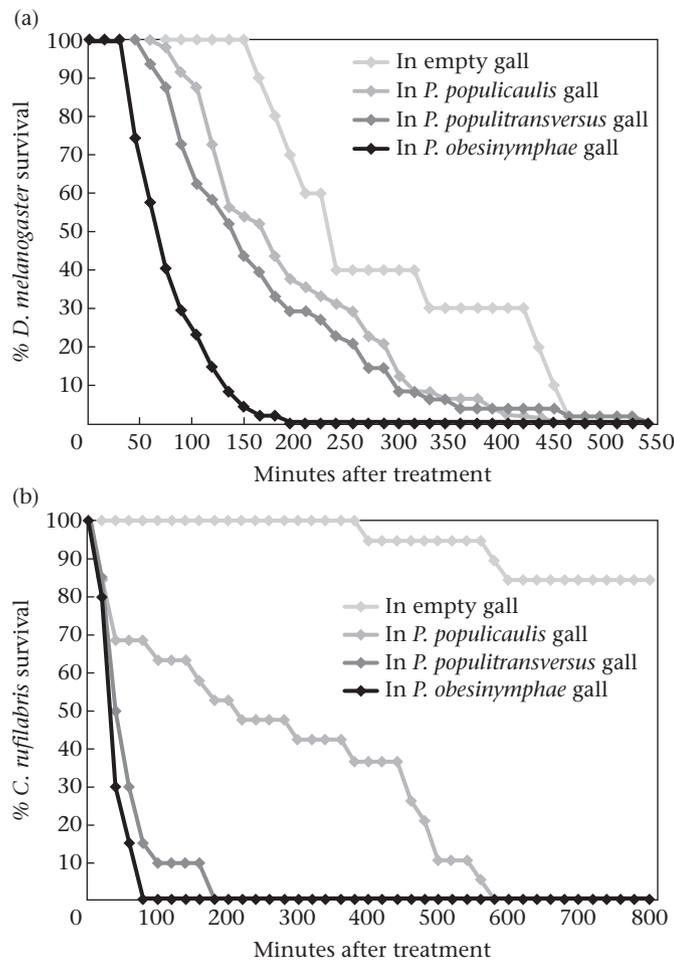

Figure 4. Average ± SE number of honeydew waste balls ejected by aphids from galls with the ostiole in the natural position (white bars; $N = 12$ for each species) and manipulated to point upwards (black bars; $N = 12$ for each species). Asterisks indicate a significant difference ($P < 0.05$).

defence, had significantly more honeydew balls remaining in the gall when the ostiole was pointing upwards relative to the other two species (Wilcoxon Lifetest: $\chi^2_5 = 52.0618$, $P < 0.0001$; Fig. 4), and *P. populicaulis* had significantly more honeydew balls remaining in the gall when the ostiole was pointing down in its natural orientation ($\chi^2_5 = 52.0618$, $P < 0.0001$; Fig. 4).

Fortress Defence

We found that the average time to death of the *D. melanogaster* larvae was negatively correlated with the nominal characterization of sociality in each species (i.e. nonsocial, weakly social or eusocial). *D. melanogaster* larvae that were introduced to the gall of the nonsocial aphid species, *P. populicaulis*, showed an average survival of 198.8 min (range 75–450 min), while larvae introduced to the galls of the weakly social species, *P. populitransversus*, showed an average survival of 175.6 min to death (range 60–540 min), and larvae in the gall of the eusocial species, *P. obesinymphae*, had an average survival of 83.6 min to death (range 45–195 min; log-rank test: *P. populicaulis* versus *P. populitransversus*: $\chi^2_1 = 0.8158$, $P = 0.3664$; *P. populicaulis* versus *P. obesinymphae*: $\chi^2_1 = 56.4564$, $P < 0.0001$; *P. populitransversus* versus *P. obesinymphae*: $\chi^2_1 = 35.6498$, $P < 0.0001$; Supplementary Video S2, Fig. 5a). Overall, regardless of species, the presence of aphids reduced survivorship of *Drosophila* larvae relative to controls placed in empty galls (log-rank test: $\chi^2_3 = 84.0620$, $P < 0.0001$; Fig. 5a).

We found similar trends using a natural predator, *C. rufilabris*. The eusocial species had the most effective soldiers (average survival of *C. rufilabris*: 45 min, range 20–80 min), while the weakly social aphid species' soldiers were the second most effective (average survival of *C. rufilabris*: 64 min, range 20–180 min), although not significantly so. The nonsocial aphid species' soldiers were the least effective (average survival of *C. rufilabris*: 266.3 min, range 20–580 min; log-rank test: *P. populicaulis* versus *P. populitransversus*: $\chi^2_1 = 12.5042$, $P = 0.0004$; *P. populicaulis* versus *P. obesinymphae*: $\chi^2_1 = 15.9044$, $P < 0.0001$; *P. populitransversus* versus *P. obesinymphae*: $\chi^2_1 = 2.5934$,

Figure 3. Average ± SE number of aphids in galls of each aphid species: *Pemphigus populicaulis* ($N = 40$); *P. populitransversus* ($N = 50$); *P. obesinymphae* ($N = 49$). Asterisks indicate a significant difference ($P < 0.05$).

Figure 5. (a) Survival of 'invading' *Drosophila melanogaster* larvae placed in an empty gall (control, $N = 20$) and in a gall containing *Pemphigus populicaulis* ($N = 48$), *P. populitransversus* ($N = 48$) and *P. obesinymphae* ($N = 48$) aphids. (b) Survival of predatory *Chrysoperla rufilabris* larvae in an empty gall ($N = 19$) and in a gall containing *P. populicaulis* ($N = 20$), *P. populitransversus* ($N = 20$) and *P. obesinymphae* ($N = 20$) aphids.

$P = 0.1073$; Fig. 5b, Supplementary Video S3). Again, *C. rufilabris* that were introduced into a gall with aphids died earlier than the controls in an empty gall (log-rank test: $\chi^2_3 = 74.9785$, $P < 0.0001$; Fig. 5b). We tested another natural predator, *Orius* spp., against the social aphid, *P. obesinymphae*, and obtained similar results. *Orius* spp. placed in a gall with aphids had a significantly earlier time to death (average: 109.5 min, range 40–220 min) compared with controls (average: 1098.9 min, range 360–1440 min; log-rank test: $\chi^2_1 = 43.5414$, $P < 0.0001$; Supplementary Fig. S1, Video S4).

To quantify defence behaviour of *Pemphigus* further, we compared the raw number of soldiers recruited by each species to attack an invader of the gall. We found that, on average, *P. obesinymphae* had significantly more soldiers per invader (18.8 soldiers) than *P. populitransversus* (8.6 soldiers) or *P. populicaulis* (3.1 soldiers) (Wilcoxon Lifetest: $\chi^2_2 = 160.7328$, $P < 0.0001$; *P. populitransversus* versus *P. populicaulis*: $\chi^2_2 = 49.7268$, $P < 0.0001$; Fig. 6).

DISCUSSION

Among their various experimental advantages, aphids offer opportunities to compare closely related species that differ in their expression of social behaviours (Stern & Foster, 1996). However,

there are few comparative studies of social behaviour in aphids that characterize the behaviours of both social and nonsocial aphids, and as yet, it is not clear how to categorize the spectrum of sociality in aphids. To describe the behaviours involved in the transition from nonsocial to social lifestyles in aphids empirically, we compared three closely related species of *Pemphigus*. We found that the two behaviours most often used to characterize sociality in aphids (defence and housekeeping) varied between eusocial and nonsocial species, but in surprising ways. An objective measure of defence (e.g. how fast do predators die?) clearly differentiated species previously described as social and those not formally recognized as social. However, nonsocial species expressed housekeeping behaviours on par with that of the eusocial species and, thus, another measure of social behaviour (the extent to which they performed maintenance of their nests) conflicts with the fortress defence standard of aphid sociality. Thus, there is not a single syndrome of social behaviour in aphids. Rather, while more thorough studies of social and nonsocial species are needed, it appears likely that the social behaviours that aphids express vary independently across species.

Life History Traits

Most *Pemphigus* aphids have two distinct host plants during the life cycle: a galling phase on the primary host plant in the genus *Populus* and a herbaceous secondary host plant. Interestingly, compared to *P. populitransversus* and *P. populicaulis*, *P. obesinymphae* has shifted its life cycle to overwinter in the roots of its secondary host plant and spend more time within the gall (Abbot & Withgott, 2004). It has been hypothesized that predation during the galling phase is one of the major ecological drivers of soldier evolution in aphids (Foster & Northcott, 1994). *Pemphigus obesinymphae* spends more of its life cycle in the gall and has the most effective soldiers (Fig. 5). The question then arises: did the shift in life cycle occur because *P. obesinymphae* evolved effective soldiers, which allowed them to stay in the gall longer, or did the shift in life cycle occur first and thus put intense selective pressure on the development of soldiers? The role of host plants in driving social evolution in aphids has been an open question for some time (Stern & Foster, 1996). To date, there are still no comprehensive studies that have investigated social evolution in aphids from the perspective of plant–insect interactions.

Altruistic Housekeeping Behaviours

Group living is inherently vulnerable to the increased risk of pathogens and disease transmission. To combat this, eusocial organisms have evolved cooperative immune responses known as social immunity (Cremer, Armitage, & Schmid-Hempel, 2007). Social immune behaviours can be prophylactic, like resin collection by ants to reduce microbial growth (Christe, Oppliger, Bancala, Castella, & Chapuisat, 2003), antimicrobial secretions by parents (Arce, Smiseth, & Rozen, 2013), or corpse removal by workers (Diez, Le Borgne, Lejeune, & Detrain, 2013) or activated in response to a pathogen, like social fever in honeybees (Starks, Blackie, & Seeley, 2000). Identifying these cooperative immune responses is key to understanding how groups reduce the risks of living together and transmitting pathogens. Aphids excrete a large volume of sugary waste or honeydew. Honeydew poses a threat to groups of aphids because they can become entrapped and drown in the watery substance (Denny, 1993). Honeydew also provides the perfect environment for microbial growth (Fokkema, Riphagen, Poot, & Dejong, 1983; Lawson, Christian, & Abbot, 2014). Free-living aphids can avoid these problems by changing feeding sites, flicking honeydew waste from the leaf, or being tended by aphids. Gall-
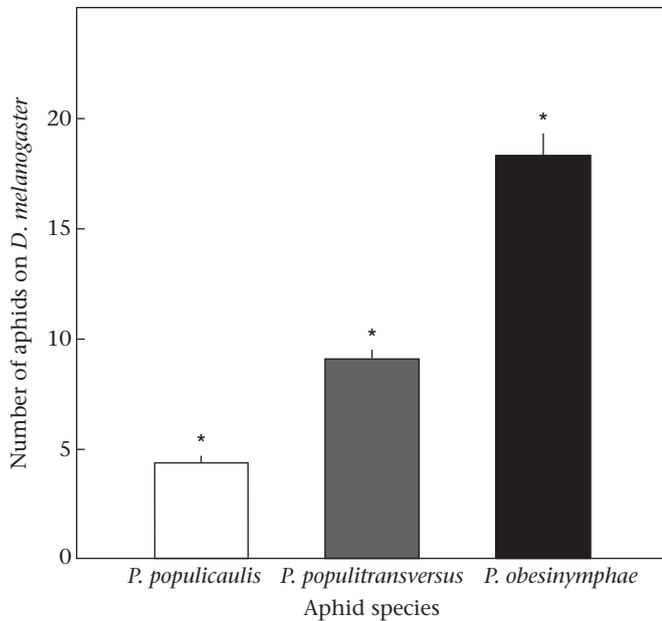
Figure 6. Average ± SE number of *Pemphigus populicaulis* (*N* = 48), *P. populitransversus* (*N* = 48) and *P. obesinymphae* (*N* = 47) that attacked an invading *D. melanogaster* during a 3 h period.

forming aphids must find other ways to avoid these challenges (Benton & Foster, 1992; Pike et al., 2002). Gall cleaning has been noted in some species, and there is even evidence of gall repair (Aoki, 1980; Aoki & Kurosu, 1989; Kurosu & Aoki, 1991; Kutsukake et al., 2009; Pike & Foster, 2004). More experimental approaches to measure altruistic housekeeping behaviours have only been thoroughly explored in *P. spyrothecae*, *P. dorocola* and *Hormaphis betulae* (Aoki, 1980; Benton & Foster, 1992; Kurosu & Aoki, 1991). To our knowledge, there have been no studies of housekeeping in species that lack soldiers.

To explore how altruistic housekeeping behaviours vary across species with and without soldiers, we compared the ability of first-instar soldiers to remove honeydew balls and exuviae waste from the gall. Past research in other species has shown that first or second instars are the predominate housekeepers (Aoki, 1980; Benton & Foster, 1992; Kurosu & Aoki, 1991). We found that all aphid species tested ejected waste from the gall. In both *P. populicaulis* and *P. populitransversus*, galls with first instars ejected significantly more honeydew balls and exuviae than galls without first instars (Fig. 2). This implies that the first instars in these species perform a majority of the housekeeping behaviours. Although there was no significant difference in fitness measurements between groups with and without first instars, our measures of fitness were rough ones, taken from laboratory populations over a short duration (Table S1). Considering that the galling length of these species varies from 3 to 9 months, this experimental period is likely not enough time to see a significant effect of the increase in honeydew balls caused by the absence of the first instars. In the highly social species, the first instars are important in the maintenance of the gall, but later instar individuals also participate in housekeeping behaviours (Video S1). Interestingly, unlike *P. obesinymphae*, in *P. populicaulis* and *P. populitransversus*, first instars do not delay development, but moult rapidly after larviposition, and their clonal groups are typically composed of individuals at various stages of development. Given that cooperative housecleaning behaviours are only expressed by the first instars in these species, whereas all instars express these behaviours in the eusocial species, the implication is that there has been an elaboration of

behaviours associated with group homeostasis in the eusocial species that is absent from other, less social species. Moreover, the presence of housekeeping in the nonsocial *P. populicaulis* indicates that selection can favour homeostatic behaviours in the absence of selection for defence, a result that is mirrored by the persistent expression of housecleaning behaviours by *P. obesinymphae* juveniles as they age, despite the fact that only the first instars express defence behaviours. Thus, in *P. obesinymphae*, defence and homeostasis can vary independently through development, and is a form of temporal polyethism not unlike that seen in other eusocial taxa like honeybees, stingless bees and ants (Mersch, Crespi, & Keller, 2013; Seeley, 1982; Sommeijer, 1984).

Another interesting finding was the significantly lower amount of honeydew ejected by *P. populitransversus* galls compared to *P. populicaulis* or *P. obesinymphae* galls (Fig. 2a). To test whether this was caused by less housekeeping activity or by less honeydew production, we manipulated the direction of the ostiole to examine the amount of honeydew produced by each species. We found that changing the direction of the ostiole led to an increase in the number of honeydew balls in the gall for all three species, and *P. populitransversus* produced significantly more honeydew balls (Fig. 4). *Pemphigus populitransversus* galls have a very different shape compared to the other two species and occur on the petiole of the poplar leaf, rather than at the base of the leaf lamina. The gall is much longer and the ostiole is not as pronounced. It is possible that there is no functional advantage to cleaning behaviour in *P. populitransversus* because the ostiole is so small; instead, they allow the honeydew to build-up within in gall. Another possibility is that the petiole is a nutritionally poor niche relative to the leaf lamina, requiring petiole-gallers to feed more than species in more nutritionally rich sections of a host plant. There is some evidence that aphid galls are resource sinks, and there may be interspecific differences in the degree to which galls concentrate plant metabolites (Larson, 1991). Finally, unusual adaptations have been described in some aphid species whose galls are closed, preventing the removal of honeydew (Kutsukake, Meng, Katayama, Shibao, & Fukatsu, 2012). Possibly, *P. populitransversus* expresses behaviours for managing honeydew that we did not account for.

Fortress Defence

Fortress defence is the defining characteristic of sociality in aphids and has been described in multiple aphid species (Aoki, Kurosu, & von Dohlen, 2001; Aoki, Kurosu, Shin, & Choe, 1999; Aoki, Kurosu, & Sirikajornjaru, 2007; Kurosu, Buranapanichpan, & Aoki, 2006; Moran, 1993; Rhoden & Foster, 2002). Surprisingly, however, there is no single means to identify fortress defence in aphids, and different studies typically use one or several life history, morphological and behavioural indices to define the presence of altruistic defence in aphids. Past measures of defence behaviours have been largely qualitative, typically noting only whether aphids placed in an arena with a predator will show aggressive behaviours. We used an objective and quantifiable measure of defence (the ability and rate at which aphids kill natural predators) that, theoretically at least, could be applied to any aphid species. Although this measure of defence quantifies the effective defence of the entire group and, thus, does not capture individual differences in the effectiveness of attack, it does allow us to measure the effective consequences of defence as an emergent property of eusocial aphid groups, which tend to be larger and composed of more aggressive soldiers than weakly social or nonsocial species, as described below.

It has been previously argued that galling aphid species display a continuously varying spectrum of altruistic defensive behaviours, from weak to highly aggressive. This is what we observed.

Pemphigus populicaulis is anecdotally a nonsocial species, but we found that its soldiers were capable of killing a natural predator, *C. rufilabris*. However, soldiers of *P. populicaulis* were less effective than those of the weakly social *P. populitransversus*, which is intermediate to the eusocial *P. obesinymphae* (Fig. 5). *Pemphigus obesinymphae* invests almost four times as many soldiers during attacks of invaders as the nonsocial *P. populicaulis* (Fig. 6). More research is needed to understand whether *P. obesinymphae* soldiers are more efficient recruiters, or whether *P. obesinymphae* galls have more soldiers available.

Another question is how these aphid soldiers are able to kill natural predators much larger than themselves. Unlike some social aphid species, the species we studied have monomorphic larvae that lack obvious morphological adaptations for defence. However, little is known about the chemical defences of aphids and whether morphological adaptations for defence predict or correlate with defensive chemistry. It has been demonstrated that soldiers in one aphid species, *Tuberaphis styraci*, use a secreted venomous protease for colony defence (Kutsukake et al., 2004).

Conclusions

What is sociality in aphids? Traditionally, the presence of a soldier caste is the defining feature of sociality in aphids. Based on this definition, *P. populicaulis* is considered a nonsocial species, but our results indicate that *P. populicaulis* does indeed have workers with modest defensive behaviours. These workers are not as effective as the weakly social *P. populitransversus* or the eusocial *P. obesinymphae* (Fig. 5). However, if housekeeping were the hallmark of sociality in aphids, much as brood care is in some other social insects, then *P. populicaulis* and *P. obesinymphae* would be considered highly social species, while *P. populitransversus* would be considered a nonsocial to weakly social species (Table 1). Our work has demonstrated that defining sociality in aphids depends critically on what trait is being measured. In addition, these data contribute to evidence suggesting that most galling aphid species, including those not traditionally described as nonsocial, may express some form of social behaviour. Most social insects outside of the Hymenoptera remain poorly studied, and even basic natural history information is often lacking, or largely anecdotal (Costa, 2006). As studies of these 'other social insects' are undertaken, the lesson from aphids is that the nonsocial species tend to be particularly poorly studied, if at all. If the goal is to understand social transitions, however, studies of nonsocial species are as necessary to comparative studies as those with social behaviour. Second, aphids illustrate the degree to which the antecedents to advanced sociality may be identified in nonsocial species. It will be revealing as studies of sociality begin to identify both the ecological as well as the more mechanistic factors that amplify or tune the latent expression of advanced social behaviours in aphids.

Acknowledgments

Supplementary Material

Supplementary material for this article is available, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2014.08.003.

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