

Evolution of larval gregariousness is associated with host plant specialisation, but not host morphology, in Heliconiini butterflies

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Abstract

Insect herbivores, such as lepidopteran larvae, often have close evolutionary relationships with their host plants, with which they may be locked in an evolutionary arms race. Larval grouping behaviour may be one behavioural adaptation that improves host plant feeding, but aggregation also comes with costs, such as higher competition and limited resource access. Here, we use the Heliconiini butterfly tribe to explore the impact of host plant traits on the evolution of larval gregariousness. Heliconiini almost exclusively utilise species from the Passifloraceae as larval host plants. Passifloraceae display incredible diversity of form, leaf shape and a range of anti-herbivore defences, suggesting they are locked in an arms race with Heliconiini larvae. By analysing larval social behaviour as both a binary (solitary or gregarious) and categorical (increasing larval group size) trait, we revisit the multiple origins of larval gregariousness across Heliconiini. We investigate whether host habitat, leaf defences and leaf size are important drivers of, or constraints on, larval gregariousness. Whereas our data do not reveal links between larval gregariousness and the host plant traits included in this study, we do find an interaction between larval host specialisation and behaviour, revealing gregarious larvae to be more likely to feed on a narrower range of host plant species than solitary larvae. We also find evidence that this increased specialisation typically precedes the evolutionary transition to gregarious behaviour. The comparatively greater host specialisation of gregarious larvae suggests that there are specific morphological and/or ecological features of their host plants that favour this behaviour.

Introduction

Many prey animals have evolved grouping behaviour in response to predation and resource availability. Lepidopteran larvae benefit from aggregating in a number of ways, ranging from increased protection from predators (Hunter, 2000; Reader and Houchuli, 2003; Greeney et al., 2012) to facilitated feeding (Clark and Faeth, 1997; Fordyce, 2003; Kawasaki et al., 2009; Campbell and Stastny, 2015; Rentería et al. 2022). However, larval gregariousness also imposes costs, such as greater competition for food resources between siblings (e.g. Despland and Le Huu, 2007; Pescador-Rubio, 2009), creating the context for possible evolutionary trade-offs. Identifying key biotic and ecological factors that frame these trade-offs may be critical for understanding the origin and evolution of gregarious behaviour. One of the most important of these ecological factors are larval host plants. As herbivores, lepidopteran larvae often have intimate coevolutionary relationships with their hosts. These plants can act as a major source of selection for larvae, for example due to their growth structure or by developing defences against herbivory that larvae must adapt to overcome (Clark and Faeth, 1997; Thaler et al., 2002; Wittstock and Gershenson, 2002; Birnbaum and Abbot, 2018; de Castro et al., 2018; Karban, 2011; Despland, 2019).

Host plant traits that may influence the evolution of larval gregariousness include their relative leaf size, anti-herbivory defences and spatial distribution. For example, the average leaf size of the host may determine its suitability for group-feeding larvae. In general, and discounting foliage density, larger leaves might provide more food to support multiple larvae, and may be an indication of greater above ground biomass (e.g. Digrado et al., 2022). Larger leaves have been shown to enhance the growth rate of young lepidopteran larvae (Potter

et al., 2012), and by providing greater amounts of resources, larger leaves might also allow gregarious larvae to reach larger body sizes. Large leaves also physically offer a wider surface area upon which larvae can collectively feed, which could be important if larvae benefit by remaining close to their group members, such as by reducing predation and parasitism risks (e.g. McClure and Despland, 2011). The spatial distribution, or density, of host plants might also vary, affecting how easily females locate suitable oviposition sites, and equally defining the risk of larvae moving between hosts if a food resource is exhausted. Females may therefore adjust their oviposition strategy in response to the relative difficulty of locating suitable hosts (Braby and Nishida, 2010), with clumped eggs giving rise to gregarious larvae (Clark and Faeth, 1998; Korb and Heinze, 2016).

Across both short- and long-term scales, plants are rarely passive in their coevolutionary relationships with larval herbivores, having evolved a variety of defences in response to being selected as hosts. Evolutionary adaptations such as tougher leaf surfaces can prevent larval feeding (see Fürstenberg-Hägg et al., 2013 for a review), and trichomes can physically prevent larvae from accessing the leaf tissue, significantly hinder movement, exude harmful substances, or may even cause integumental injuries (Gilbert, 1971; Fürstenberg-Hägg et al., 2013; Despland, 2019). The evolution of toxins also helps plants to escape herbivory from many generalists (Wittstock and Gershenson, 2002; Engler-Chaouat and Gilbert, 2007; Birnbaum and Abbot, 2018). Furthermore, plants which have evolved toxins often also display more immediate responses to attack, such as the release of these concentrated toxins into sites of feeding damage (Denno and Benrey, 1997; Karban, 2011). These host plant defences, and the need to overcome them, are thought to be a main promoter of larval aggregation in some systems (Clark and Faeth, 1997; Denno and Benrey, 1997; Fordyce and Agrawal, 2001; Kawasaki et al., 2009; Despland, 2019; Rentería et al., 2022). For example, some larvae will meticulously remove leaf trichomes to reduce their harmful impact (de Castro et al., 2018), but this is likely to be a costly task for an individual. Some gregarious larvae are well-equipped to deal with trichomes, and collectively cover them in silk to avoid contact (e.g. Rathcke and Poole, 1975; Despland, 2019; Despland, 2021). Additionally, collective feeding is thought to benefit larvae against their host's toxin release response if they can completely consume the leaf before it is flooded with toxins (Denno and Benrey, 1997).

Here, we use the Heliconiini butterfly tribe as a model system to study the influence of specific host plant traits on the evolution of larval gregarious behaviour. All Heliconiini larvae feed on vines from the Passifloraceae family (de Castro et al., 2018), which offers a shared ecological context within which specific trait differences can be interrogated. Passifloraceae are highly diverse, varying widely in their overall structure and defences against herbivory, such as egg mimicking structures to deter oviposition, extrafloral nectar rewards to attract predatory ants, and toxic chemical components in their tissues (de Castro et al., 2018). These chemicals form an important line of defence against herbivory from generalist species and, perhaps as a result, some Heliconiini have been driven into specialising on small numbers of hosts. Heliconiini larvae have evolved resistance to their host's toxins, often in correlation with increased specialisation (Engler-Chaouat and Gilbert, 2007; Merrill et al., 2013; de Castro et al., 2021), and the ability to incorporate these toxins into their own chemical defences (Engler-Chaouat and Gilbert, 2007; Arias et al., 2016; de Castro et al., 2021). Additionally, larval social behaviour varies across the Heliconiini, even between very closely related species, with repeated shifts to grouped egg laying and gregarious larvae (Beltran et al., 2007; McLellan et al., 2023). Although little is known about the behavioural mechanisms supporting these aggregations, at least some gregarious Heliconiini are trail followers (Pescador-Rubio et al., 2011), suggesting these transitions reflect behavioural adaptations in larvae rather than simple variation in female egg laying. This variation, coupled with tribe-wide estimates of the phylogenetic structure of the Heliconiini (Kozak et al., 2015; Cicconardi et al., 2022), positions these butterflies as a highly useful system with which to study behavioural evolution in response to host plant ecology.

Here, we revisit the evolution of larval gregariousness in Heliconiini, taking a phylogenetic comparative approach to identify where transitions to larval gregariousness have taken place across the phylogeny. Then, by exploring variation in host plant use between the two behavioural phenotypes, we test hypotheses regarding the host traits that shape the evolution of gregarious behaviour. In particular, we ask i) do gregarious larvae use a narrower range of host plant species than solitary larvae? ii) Do aggregated larvae occur only on hosts

with specific traits? iii) Is host plant leaf size a key constraint on the evolution of gregarious larvae and their late instar body size? And iv) do commonly used host plants occur in particular contexts or lack particular defences, rendering them more accessible to predation?

Methods

i) Phylogenies and species lists

We obtained two lists of 52 and 75 butterfly species within the Heliconiini tribe from phylogenies used in Cicconardi et al. (2022) and Kozak et al. (2015) respectively. For our primary analyses we use Kozak et al.’s phylogeny (Figure 1A), as this incorporates a larger total number of species. However, we also repeated all analyses using Cicconardi et al.’s phylogeny (see Appendix for these results), as this is based on a greater amount of molecular data, and has some minor topological differences, but contains fewer species.

ii) Larval and host plant data

We collected data on the larval social behaviour, Passifloraceae host species and adult wingspan of the Heliconiini included in each phylogeny (all data with references can be viewed in online repository). Data on larval social behaviour, recorded as a binary variable (0 = solitary, 1 = gregarious) were based on previous classifications of egg clutch size range (Brown, 1981; Beltran et al., 2007), with gregariousness recorded for any species with a range maximum [?] 10 (as per Beltran et al.’s 2007 categorisation). We additionally analysed social behaviour as a categorical variable (0/G1/G2/G3) as per records of four levels of increasing ‘sociability’ based on clutch size (Brown, 1981): Solitary species were recorded as zero. Gregarious level one (G1) species were any with a minimum of 10 eggs in their clutch size range, and those with median clutch sizes between 10.5 and 24. Finally, gregarious level two (G2) and three (G3) species were those with median clutch sizes between 25 and 30, and greater than 30 respectively. Use of Passifloraceae host species were mainly taken from published sources (Benson et al. 1975; Kozak, 2016; Young et al. 2023). For a small number of species, all in the ‘Neruda’ clade of the genus *Heliconius*, we only recorded the host at the genus level (*Dilkea*) as we lacked species-level data, this meant we could not confirm how many separate species within this genus are used as hosts, despite there being 13 accepted species within *Dilkea* (POWO, 2022). The four species in the Neruda clade occur at lower densities than other *Heliconius* and lack some derived features of the genus. Re-running the analyses with these genus-level entries omitted had no meaningful effect on the results (Table A1).

Trait data on the Passifloraceae hosts were taken from two main sources (Benson et al., 1975; Ulmar and MacDougal, 2004). To test for the effect of host habitat on larval behaviour, we recorded four distinct, main habitats (forest interior, forest edge, open areas and humid glades), taken exclusively from Benson et al. (1975). To test if the presence of leaf trichomes predicts larval gregariousness, we recorded the vestiture (the presence or absence of leaf trichomes) of hosts as a binary variable (0 = glabrous (lacking trichomes), 1 = pubescent (possessing trichomes)), where leaves listed as ‘nearly glabrous’ were recorded as glabrous. A plant’s growth habit may give an indication of its size, for example a tree is likely to be bigger than a shrub, and it is possible that larger plants are better at supporting gregarious larvae. However, the Passifloraceae included in this study show a considerable lack of diversity in growth habit (only ~ 9% are not vines), making this trait an uninformative proxy for comparing overall host size. Instead, we focused on leaf size as an indicator of above ground biomass (e.g. Digrado et al., 2022). To determine whether larger leaves predict gregarious larvae and/or larger gregarious larvae, we recorded the mature leaf size of each Passifloraceae in our dataset, given by Ulmar and MacDougal as separate ranges (minimum and maximum) of leaf length and width (both cm). Here, we first calculated the median value of these ranges, then multiplied one median by the other to gain an estimate of the median leaf surface area, assuming an idealised leaf shape, which was used as a final value for ‘leaf size’(de Luna Souto et al., 2017). We acknowledge that, given the leaf shape diversity in *Passiflora*, leaf area is likely a crude estimate of overall leaf tissue available to larvae.

Finally, we obtained adult wingspan data from recordings of specimens collected in the field (Couto et al. 2022) and images from Butterflies of America (Warren et al., 2016). Where available, we measured images of five separate specimens, then calculated the mean wingspan (mm) for that species. Adult wingspan was

used as a proxy for final instar larval size, as this has previously been found to be reliable (Nilsson and Forsman, 2003; Hebberecht et al., 2022; McLellan et al., 2023). This allowed us to test for a relationship between approximate larval size and host leaf size, to assess whether larger leaves are generally required to support larger larvae. Leaf size and wingspan data were log transformed for all analyses.

iii) Behavioural transitions

All analyses were performed using R v. 4.1.2 (R Core Team, 2013). We determined the phylogenetic signal of larval social behaviour, as estimated by Pagel’s λ (Pagel 1999), using both the ‘fitDiscrete’ function in *geiger* (Pennell et al. 2014) and ‘phylosig’ from *phytools* (Revell, 2012). We also used ‘fitDiscrete’ to compare models of evolutionary transition rates between solitary and gregarious states throughout the phylogeny. We used a likelihood ratio test to compare the null model, which assumes equal between-state transition rates across the tree, against the all-rates-different model, which assumes the between-state transition rate is different in either direction. We then used the ‘make.simmap’ function in *ape* (Paradis and Schliep 2018) and the best-fitting transition rate model to estimate the social behaviour character state at each node, and the number of independent transitions to gregariousness throughout the phylogeny. We constrained the root node to the solitary character state based on estimations from a larger butterfly phylogeny that the last common ancestor of Heliconiini was solitary with high (67%) confidence (McLellan et al., 2023). This improved confidence in estimations around basal nodes. When analysing categorical social behaviour, we used the ‘ace’ function in *ape* to estimate the most likely character states at each node. This function does not allow the option to constrain the root node, nor the use of the ‘all rates different’ evolution model with a multi-levelled, discrete variable. We therefore performed the analyses under the ‘equal rates’ model. Additionally, we performed phylogenetic pathway analyses (PPA) using the package *phylopath* (von Hardenberg and Gonzalez-Voyer, 2013) on categorical behavioural data and host plant usage data. Our first model set exclusively tested social behavioural evolution, to test the hypothesis that increasing levels of gregariousness evolves from solitariness in a linear pattern. We included the number of larval host plants used in our second model set to investigate the order in which increased host specialisation and transitions to gregariousness typically evolve.

iv) Tests of correlated evolution

We performed tests for correlated evolution between larval social behaviour and the other larval and host plant traits, whilst controlling for phylogenetic effects, using MCMCgmm (Hadfield 2010). We ran MCMC models for 5.1 million iterations, with a 0.1 million burn-in and sample storage frequency of every 500 iterations, with significance of the model calculated as the probability of the parameter value being different from zero (P_{MCMC}). We also report each model cofactor’s posterior mean (P-mean) and its 95% confidence intervals (CI). All analyses were performed using uninformative, parameter expanded priors for the random effect (G: V = 1, nu = 1, alpha.mu = 0, alpha.V = 1,000; R: V = 1, nu = 0.002) and default priors for the fixed effects. Finally, the Heliconiini dataset on host plant use could potentially contain some uncertainty as it combines records from geographically disperse Heliconiini species. Host plant use can vary across populations of single Heliconiini species (e.g. Merrill et al., 2013), potentially leading to overestimations in host use numbers for some species when data are combined over wide geographical ranges. We therefore also performed a more taxonomically limited analysis of a well studied community of 14 Heliconiini and nine *Passifloras* species in Panama (Merrill et al., 2013), as described above.

Results

i) Many origins of gregarious larvae in Heliconiini

Overall, we recorded 23 gregarious species (Figure 1A), out of the 75 Heliconiini included in Kozak et al. (2015). Our model estimated there have been seven independent transitions to gregariousness across the Heliconiini phylogeny (under the ‘all rates different model’, model comparison: $X^2(1) = 4.812$, $p = 0.028$, transition rate = 0.024). Despite this pattern of convergent evolution, the phylogenetic signal of social behaviour is estimated to be very strong ($\lambda = 1$), likely reflecting the general tendency for transitions to occur at the base of specious clades. Transitions between solitary and gregarious states are most likely to occur at

different rates, with 11 reversals from gregarious larvae back to solitary larvae across the phylogeny (transition rate = 0.078). This dynamic turnover of social behaviour suggests Heliconiini oviposition behaviour is likely responding to a range of selection pressures.

ii) Gregarious larvae use specialised host plant ranges, but not morphologies

Solitary larvae feed on a greater variety of host species than gregarious larvae (P-mean = -0.920, 95% CI -1.622 - -0.235, $P_{MCMC} = 0.015$, Figure 1B). After omitting *Passiflora* hosts used by fewer than four larval species from the data, we found that *P. pedata*, which is host to five Heliconiini species, is more likely to be used by solitary larvae. Additionally, we found that more frequently used hosts by all larvae tend to grow in forest edge habitats (P-mean = 2.741, 95% CI 0.878 - 4.451, $P_{MCMC} = 0.004$, Table A2). Contrary to our predictions, we did not identify any interactions between specific host plant traits, including leaf size, and larval social behaviour (Table A2, Figure 2). We also found no interaction between adult wingspan and host leaf size across gregarious larvae (P-mean = -0.022, 95% CI -0.572 - 0.532, $P_{MCMC} = 0.951$).

iii) Gregariousness evolves semi-linearly and is preceded by host specialisation

Estimates from our Phylogenetic Path Analysis (PPA) revealed that, from a solitary ancestor, G1 and G2 are both equally likely to evolve next (pathway coefficients: solitary to G1 = -4.303; solitary to G2 = -4.146). Our model also supports the pathway in which G1 evolves before G2 (G1 to G2 = -2.652), and G2 precedes the evolution of G3 (G2 to G3 = 5.117, Figure 3A, Table A3). When host plant number is included in the model set, a solitary ancestor is estimated to precede G1 (solitary to G1 = -3.580) and there is strong support for G2 preceding G3 (G2 to G3 = 5.693), but these character state pairs are independent of one another. Our second model also suggests that a decrease in host use number precedes transitions to G1 (host number to G1 = -0.394, Figure 3B, Table A3).

iv) A focused assessment in a single community

Pooling data from multiple Heliconiini populations across a broad geographical range has the potential to skew host plant use data. Therefore, we repeated our analyses on a less species-diverse, localised population of Heliconiini with an extensive history of ecological study. Using these data, we found no interaction between larval social behaviour and the number of different *Passiflora* species they feed on (P-mean = -0.925, 95% CI -2.673 - 0.637, $P_{MCMC} = 0.261$). However, we support our finding of a lack of interaction between specific host plant traits and larval behaviour (Table A4), however we did again observe that hosts which grow in forest edge habitats tend to be more frequently used by larvae (P-mean = 0.931, 95% CI 0.225 - 1.621, $P_{MCMC} = 0.027$, Table A4).

Discussion

Larval Lepidoptera have close evolutionary relationships with their host plants, where the phenotype of either organism often influences the traits of the other. Sometimes, behavioural evolution in larvae is not just in response to predation pressure, but is instead mainly driven by an antagonistic relationship with their hosts. Our findings support those of Beltran et al. (2007) in that there is likely to have been multiple independent evolutions to gregariousness across the Heliconiini. Larval gregariousness is mostly concentrated in distinct clades across the phylogeny (Figure 1A). This pattern is expressed quantitatively by the high phylogenetic signal of larval social behaviour. Additionally, our data reveal that larval gregariousness, measured by proxy of egg clutch size, increases over evolutionary time in a semi-linear pattern, whereby transitions to small clutches from single eggs tend to precede much larger clutches (Figure 3A). This suggests that there may be physiological constraints to females laying large clusters of eggs, which are incrementally overcome by increasing smaller clutch sizes over evolutionary time, and/or a selection pressure feedback loop which promotes larger clutches once gregariousness evolves.

Our analyses of host plant ecology support only one of our hypotheses, that gregarious species tend to be more specialised. Our data show that Heliconiini species with solitary larvae tend to feed on a greater variety of host species than gregarious larvae (Figure 1B). Unless they deposit their entire egg load onto one plant, females of species with solitary larvae will necessarily visit more hosts than egg-clustering species

to lay a comparable number of eggs. Thus, if females need to visit a higher number of oviposition sites, potentially across multiple separate host plants, they may benefit from being comparatively less selective of these hosts. From solitary, generalist larvae, our data suggest that increased host specialisation evolves before the transition to gregariousness. A possible explanation for this evolutionary pathway is that the host plants that are utilised by species with gregarious larvae are of high nutritional quality, and are better positioned to support groups of larvae. This is supported by evidence that females of other butterfly species with gregarious larvae preferentially oviposit on higher quality hosts (Schäpers et al., 2016). We note that the findings from our focused assessment indicate that, at the local level, host plant specialisation does not significantly differ between solitary and gregarious Heliconiini, despite a similar interaction coefficient to the main model. This lack of significance is likely an effect of low power, indicated by the larger CI range in this second model, and the low numbers of hosts reportedly used by all species across this dataset.

In general, one mechanism proposed to allow host specialists to escape competition is through the evolution of toxin resistance to enable the colonisation of new resources. The *Passiflora* have robust chemical defences, and this toxicity can vary across species (de Castro et al., 2018, 2019). However, co-evolution alongside their hosts allows specialised larvae to minimise the fitness costs associated with metabolising their hosts' toxins, to a greater degree than achieved by closely related generalist species (e.g. Engler-Chaouat and Gilbert, 2007; Merrill et al., 2013; de Castro et al., 2021). Increased toxicity resulting from host specialisation might explain our finding that this behaviour evolves before gregariousness for most Heliconiini (Figure 3B), given that larval toxicity most likely precedes transitions to gregariousness across the wider butterfly phylogeny (McLellan et al., 2023). Maintaining this specialisation may also be a response to high inter-specific competition for food resources (e.g. Merrill et al., 2013), as minimising additional, inter-specific competition is likely to be disproportionately important to grouped larvae. Although we were unable to account for plant chemistry in the current study, this explanation is supported by evidence that both gregarious and specialist *Heliconius* species are more toxic than solitary and generalist species respectively (Arias et al., 2016). *Heliconius* specialists are better at sequestering their host's toxins than generalists but may be worse at synthesising their own (Engler-Chaouat and Gilbert, 2007). This could create an evolutionary feedback loop, whereby specialists become 'locked in' to their host, or else suffer reduced defences.

Despite evidence of an effect of host plant diversity on gregarious behaviour, none of the specific host plant traits examined in this study act as predictors of Heliconiini larval gregariousness. Whereas host plant morphology is thought to influence the evolution of other larval traits, such as anti-predator colour strategy (Prudic et al., 2007), our results do not show similar influences on social behaviour. First, we predicted that hosts with larger leaves would, on average, be preferred by gregarious larvae. Remaining as a closely aggregated group on a single plane has been shown to offer increased protection to larvae nearer the centre of the aggregation (McClure and Despland, 2011). Thus, our assumption was that leaves with larger surface areas would provide better 'stages' for such groupings. Additionally, evidence suggests that lepidopteran larvae develop faster on larger leaves (e.g. Potter et al., 2012), so it plausible that they might grow larger too. We found no effect of leaf size on larval social behaviour, suggesting that even small leaves may be big enough to support groups of larvae if larvae are small or the groups do not contain many individuals. Similarly, we found no evidence to suggest that larger leaves predict larger gregarious larvae, possibly because food availability may not depend on leaf size if leaves are numerous and easy to travel between. Additionally, we recorded leaf size based on mature leaf data (Ulmar and MacDougal, 2004), yet larvae may preferentially feed on young leaves (e.g. Peterson, 1987) given the potential growth benefits available (e.g. Coley et al., 2006). Our findings may nevertheless indicate that other factors, such as foliage density and ease of travel between leaves, require consideration if resource availability is to be more accurately measured.

We also predicted an evolutionary link between the presence of host leaf trichomes and larval gregariousness. This was because group-feeding can aid larvae in overcoming certain host plant defences, and in some cases may even be necessary such as for negating effects of feeding-induced toxins and leaf toughness (Denno and Benrey, 1997; Fordyce, 2003; Kawasaki et al., 2009; Despland, 2019; Rentería et al., 2022). Our hypothesis was that these feeding facilitation benefits may contribute towards a form of behavioural character displacement, where gregarious larvae specialise on well-defended hosts that solitary species struggle to feed on. Indeed,

some host plant leaf trichomes act as formidable defences against larval herbivores (e.g. Gilbert, 1971; Despland, 2019) and in one system, aggregation is thought to have evolved as a response to overcome this defence (Despland, 2019). However, we found that the presence or absence of *Passiflora* leaf trichomes has no influence on larval social behaviour in the Heliconiini. This may be because of a lack of specificity in available vestiture data, meaning we could only record vestiture in binary format and lacked information on the length and density of trichomes on most of the pubescent plants. Other useful trichome data, particularly their structure (whether they are hooked, glandular or neither), are also absent from the literature. These features are likely to be important determinants of how difficult trichome defences are for larvae to overcome (Fürstenberg-Hägg et al., 2013; Despland, 2019). Alternatively, it may simply be the case that, in most cases, aggregating does not improve larvae's ability to overcome trichomes to an extent that it is selected over solitary feeding.

Finally, we expected larval behaviour to vary according to their host plant's (and by extension their own) main habitat, given the potential ecological differences between them. While we are missing habitat data for a number of host species in this study, overall, we found no evidence that habitat predicts larval behaviour. Our inclusion of habitat type was based on the assumption that it may act as a proxy for ecological factors which potentially influence larval social behaviour, such as host spatial distribution (Young, 1983; Braby and Nishida, 2010). Our negative result may indicate that this assumption is not valid. However, in both our geographically broad and focused datasets we observed that hosts growing on the edges of forest habitats are favoured by Heliconiini in general, regardless of social behaviour. It is possible that there is some aspect of this habitat that ovipositing females favour over others, although we cannot rule out a bias in how these data are recorded, such as edge habitats being easier to access than forest interiors.

In summary, larval gregariousness is widespread across the Heliconiini and has evolved repeatedly, however the specific ecological drivers of this behaviour remain unclear. Variation in host specialisation between solitary and gregarious larval Heliconiini suggests that there are certain host traits that promote aggregation, however available data has not led to their identification. We suggest a number of ecological factors which we could not include in our analyses may be relevant. First, host toxicity can vary widely (de Castro et al., 2018, 2019; Mattila et al., 2021), but the chemical defences of the larvae which sequester these toxins may be a strong selective driver of which species can afford to aggregate (Ruxton and Sherratt, 2006; McLellan et al., 2023). We did not include plant chemistry in our analyses, but this may have given us a better understanding of why larvae specialise onto certain hosts, for example to escape competition from generalists. Currently, however, data on toxicity variation across populations of larvae and their hosts is lacking. Second, host spatial density, and whether it varies between habitats, is an important factor missing from our data. We attempted to capture this by testing for an effect of host habitat on larval behaviour, on the assumption that the categories capture structural variation in the forest. However, direct data on how those habitats, or plants within them, might differ ecologically is lacking. As such, larval gregariousness may arise in response to ecological specialisation by the host plants, if the preferred host is sparsely distributed and difficult to locate, benefitting females which lay their eggs in clusters (Young 1983; Braby and Nishida 2010). Data on the relative spatial distributions of Heliconiini hosts would need to be collected if this hypothesis is to be tested, which is a challenging endeavour. Nevertheless, we have identified that increased host specialisation frequently occurs before the evolution of gregarious behaviour in Heliconiini larvae. This suggests that there are key host plant traits which may predict this behaviour, which require further study to identify.

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Figure legends

Figure 1 . A) The Heliconiini phylogeny, adapted from Kozak et al. (2015), showing larval social behaviour (solitary = grey tip points, gregarious = blue tips) and the number of separate Passifloraceae used by each species as larval host plants (1-57 represented by increasing tip point size). B) The frequency of solitary (grey bars) and gregarious (blue bars) larvae which use each number of separate Passifloraceae host species. The majority of gregarious species use < 20 hosts. C) Image examples (with credit) of various Heliconiini larvae. Clockwise from top-left: *Agraulis vanilla* , *Heliconius hewitsoni* , *Heliconius doris* , *Eueides isabella* .

Figure 2 . The frequencies at which Passifloraceae with certain traits are used by both solitary (grey bars and points) and gregarious (blue bars and points) Heliconiini larvae. A) Larval use of host plants separated by their main habitat. B) Larval use of hosts separated by their vestiture (presence or absence of leaf trichomes). C) larval use of host plants separated by their median mature leaf size.

Figure 3. Results from phylogenetic pathway analyses (PPA) in which directional evolutionary relationships between traits are estimated. Arrows show the direction of evolutionary interactions, the values next to arrows display the pathway coefficient (strength of the estimated relationship). Between categories of social behaviour, negative coefficients (red arrows) indicate that a loss of the parent trait precedes a gain of the child trait. Positive coefficients (blue arrows) indicate that a transition to the parent trait precedes a subsequent transition to the child trait. A) Output from the model looking only at larval social behaviour, showing strong support for the pathway in which initial solitariness precedes transitions to both gregarious levels 1 and 2, which in turn occur before the evolution of gregarious level 3. B) Output from the model including the degree of larval host specialisation (number of host plants used). With host use count as the parent trait, the negative coefficient indicates that a decrease in the number of host plants used precedes a transition to gregariousness.

Data accessibility statement

All data files and R scripts used in this study, along with citations to data sources, are available from Zenodo repository, DOI: 10.5281/zenodo.8086421

Additional information is provided in the Appendix.

Competing interests statement

The authors declare no competing interests.

Author contributions

S.H.M. conceived the research with additional contribution from C.F.M.. C.F.M. collected the data and performed the main analyses with assistance from S.H.M.. C.F.M. wrote the first draft with subsequent contributions from S.H.M..

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Appendix

Appendix methods

We collected data on larval social behaviour from Brown (1981) in binary, categorical and continuous formats. Brown provides the clutch size range of each Heliconiini species, which we used as a proxy for our categorical and continuous data on larval gregariousness. Categorical social behaviour was recorded as four levels (solitary, G1, G2, G3), as explained in the main methods. We calculated the median clutch size from the ranges given in Brown (1981), these median clutch sizes were then used for the continuous data. We performed additional transition rate analyses on these categorical and continuous data.

Before testing for evolutionary relationships between larval social behaviour and various host plant traits, we performed a count on the host data to reveal how many different larval species use each plant as a host. Using these count data, we omitted hosts used by fewer than four separate larvae to create a more robust test for larval social behaviour against individual host species. We also used these count data to determine which hosts are used more frequently by larvae (Table A5).

Appendix results

i) Main analyses using an alternate phylogeny

We performed all our main analyses on an additional, separate Heliconiini phylogeny (Cicconardi et al., 2022). Overall, we recorded 19 gregarious species, out of 57 included in Cicconardi et al. (2022). Our model estimated eight independent transitions to gregariousness, and the phylogenetic signal of social behaviour is estimated to be very strong ($\lambda = 1.0$). Transitions between solitary and gregarious states are most likely to occur at equal rates ($X^2(1) = 2.146, p = 0.143$, transition rate = 0.033), with four reversals from gregarious larvae back to solitary larvae across the phylogeny. Using categorical social behaviour data, the ‘all rates different’ model is better fitting (model comparison: $X^2(1) = 29.014, p < 0.001$), with the highest transition rate estimated to be from G2 to G3 (0.683 vs next highest G3 to G2 = 0.539).

As reported in our main analysis, solitary larvae feed on a greater variety of host species than gregarious larvae (P-mean = -0.855, 95% CI -1.673 – -0.083, $P_{MCMC} = 0.039$). After omitting *Passiflora* hosts used by fewer than four larval species from the data, we found no evidence of an interaction between larval social behaviour and any host species. Additionally, we found that more frequently used hosts by all larvae tend to grow in woods edge habitats (P-mean = 2.989, 95% CI 1.322 – 4.681, $P_{MCMC} = 0.001$, Table A6). Similar to our main results, we did not identify any interactions between specific host plant traits, including leaf

size, and larval social behaviour (Table A6). We also found no interaction between adult wingspan and host leaf size across gregarious larvae (P-mean = -0.031, 95% CI -0.586 – 0.552, P_{MCMC} = 0.914).

ii) Transition rate analyses of categorical and continuous social behaviour data

When analysed as a categorical variable, we recorded 10 G1, five G2 and eight G3 species out of 75 included in Kozak et al. (2015). The phylogenetic signal is strong ($\lambda = 0.829$) and not significantly different from 1.0 ($X^2(1) = 3.044$, $p = 0.081$). Transitions between behavioural states are estimated to occur at different rates ($X^2(11) = 27.548$, $p = 0.004$), with the highest transition rate estimated to be from G2 to G3 (transition rate = 0.320 vs next highest G1 to solitary = 0.177). We did not constrain the root node in our analysis of categorical social behaviour, but solitariness was estimated as the most likely ancestral state (likelihood of 0.950 for solitariness vs 0.018 next likeliest).

When analysed as a continuous variable, larval social behaviour is estimated to follow an Ornstein-Uhlenbeck model of evolution (comparison with BM model: $X^2(1) = 8.800$, $p = 0.003$), with a relatively weak mean reverting force ($\alpha = 0.104$) towards the optimum clutch size ($z_0 = 11.290$). Again, the phylogenetic signal is strong ($\lambda = 0.862$) and not significantly different from 1.0 ($X^2(1) = 1.587$, $p = 0.208$).

Appendix tables

Table A1. Coevolutionary interactions between larval social behaviour and host plant traits from Kozak et al.'s (2015) phylogeny, where the host genus *Dilkea* has been omitted from the data. Significant interactions are denoted by an asterisk.

Trait	Levels	P-mean	95% CI		P_{MCMC}
			Lower	Upper	
Host use count	N/A	-0.783	-1.507	-0.078	0.035*
Habitat	Edge	-109.074	-338.546	42.085	0.163
	Glade	-10.849	-59.826	20.957	0.563
	Interior	5.811	-39.564	55.802	0.750
	Open	-15.566	-82.591	23.970	0.619
Vestiture	Glabrous/pubescent	-1.248	-32.891	26.979	0.888
Leaf size	N/A	1.053	-18.640	19.467	0.879

Table A2. Two separate model sets testing the coevolutionary interactions between larval social behaviour and host plant traits using Heliconiini larvae included in Kozack et al.'s (2015) phylogeny. Significant interactions are denoted by an asterisk. Model set A: larval social behaviour against host traits. Model set B: larval host plant use frequency against host traits. Positive coefficients (P-mean) indicate that host species with the given trait are used by many larvae, whereas negative coefficients indicate the host is less frequently used.

Host trait	Levels	P-mean	95% CI		P_{MCMC}
			Lower	Upper	
Model set A					
Habitat	Edge	-92.001	-232.577	46.540	0.171
	Glade	-3.052	-43.149	28.011	0.815
	Interior	8.197	-22.514	43.876	0.614
	Open	-6.892	-49.978	28.816	0.793
Vestiture	Glabrous/pubescent	-0.489	-17.046	13.641	0.914
Leaf size	N/A	2.113	-21.633	27.344	0.871
Model set B					
Habitat	Edge	2.741	0.878	4.451	0.004*

Host trait	Levels	P-mean	95% CI	95% CI	P _{MCMC}
	Glade	0.039	-0.205	0.278	0.747
	Interior	-0.037	-0.315	0.235	0.806
	Open	0.008	-0.321	0.333	0.964
Vestiture	Glabrous/pubescent	-0.015	-0.207	0.180	0.880
Leaf size	N/A	-0.004	-0.311	0.297	0.983

Table A3. Pathway coefficients from the averaged best models in two separate PPA model sets, also given are the standard error (SE) and confidence intervals (CI) of each estimated pathway. Traits are solitariness (Sol), three increasing levels of gregariousness (G1-3) and the number of host plant species used by larvae (HN).

Model set	Pathway	Path coefficient	SE	CI	CI
				lower	upper
Social behaviour only	Sol -> G1	-4.303	1.536	-5.125	-1.837
	Sol -> G2	-4.146	1.576	-4.995	0.072
	G1 -> G2	-2.652	1.646	-3.474	0.192
	G1 -> G3	0.491	0.340	0.333	2.836
	G2 -> G3	5.117	2.949	0.424	5.117
Social behaviour & Host number	HN -> Sol	0.397	0.308	-0.207	1.002
	HN -> G1	-0.394	0.482	-1.339	0.551
	HN -> G2	0.029	0.262	-0.484	0.542
	Sol -> G1	-3.580	1.360	-6.246	-0.914
	G2 -> G3	5.693	3.131	-0.444	11.830

Table A4. Two separate model sets testing the coevolutionary interactions between larval social behaviour and host plant traits using data from a community of 14 Heliconiini and nine *Passiflora* species in Panama (Merrill et al., 2013). Significant interactions are denoted by an asterisk. Model set A: larval social behaviour against host traits. Model B: larval host plant use frequency against host habitat. Positive coefficients (P-mean) indicate that host species with the given main habitat are used by many larvae, whereas negative coefficients indicate the host is less frequently used.

Host trait	Levels	P-mean	95% CI	95% CI	P _{MCMC}
			Lower	Upper	
Model set A	Model set A	Model set A	Model set A	Model set A	Model set A
Habitat	Edge	-109.00	-477.39	191.61	0.430
	Glade	51.98	-239.14	340.95	0.683
	Open	-628.03	-1454.66	180.88	0.118
Vestiture	Glabrous/pubescent	-39.58	-205.41	93.53	0.665
Leaf size	N/A	-247.7	-880.9	350.5	0.397
Model B	Model B	Model B	Model B	Model B	Model B
Habitat	Edge	0.931	0.225	1.621	0.027*
	Glade	0.059	-1.179	1.218	0.911
	Open	-1.462	-4.786	1.775	0.381

Table A5. The *Passiflora* host species that are used by the greatest number of Heliconiini larvae included in Kozak et al.'s (2015) phylogeny. Included are the number of larval species which use each host and the

host traits.

Species	No. larvae used by	Growth habit	Habitat	Vestiture	Leaf area (cm)
<i>P. laurifolia</i>	21	Vine	Edge	Glabrous	51.75
<i>P. vitifolia</i>	18	Vine	Edge	Pubescent	115
<i>P. edulis</i>	16	Vine	Edge	Glabrous	225
<i>P. auriculata</i>	15	Vine	Edge	Glabrous	70
<i>P. oerstedii</i>	15	Vine	Edge	Glabrous	63
<i>P. biflora</i>	14	Vine	Glade	Glabrous	33.75
<i>P. nitida</i>	14	Vine	Edge	Glabrous	140
<i>P. capsularis</i>	13	Vine	Edge	Pubescent	60.13
<i>P. foetida</i>	13	Vine	Open	Pubescent	80.75
<i>P. suberosa</i>	13	Vine	Glade	Pubescent	74.79
<i>P. caerulea</i>	12	Vine	Edge	Glabrous	126
<i>P. coccinea</i>	12	Vine	Edge	Pubescent	50
<i>P. quadrangularis</i>	12	Vine	Edge	Pubescent	67.5
<i>P. quadriglandulosa</i>	12	Vine	Edge	Glabrous	32
<i>P. rhamnifolia</i>	12	Shrub	Interior	Pubescent	44
<i>P. sidaefolia</i>	12	Vine	Edge	NA	NA

Table A6. Two separate model sets testing the coevolutionary interactions between larval social behaviour and host plant traits using Heliconiini larvae included in Cicconardi et al.'s (2022) phylogeny. Significant interactions are denoted by an asterisk. Model set A: larval social behaviour against host traits. Model set B: larval host plant use frequency against host traits. Positive coefficients (P-mean) indicate that host species with the given trait are used by many larvae, whereas negative coefficients indicate the host is less frequently used.

Host trait	Levels	P-mean	95% CI	95% CI	P _{MCMC}
			Lower	Upper	
Model set A					
Habitat	Edge	-73.302	-225.487	83.437	0.293
	Glade	-6.971	-42.794	26.520	0.719
	Interior	3.366	-35.381	44.157	0.828
	Open	-7.981	-49.500	30.254	0.735
Vestiture	Glabrous/pubescent	-6.667	-42.064	26.752	0.709
Leaf size	N/A	3.472	-16.374	27.147	0.796
Model set B					
Habitat	Edge	2.989	1.322	4.681	0.001*
	Glade	0.047	-0.204	0.326	0.727
	Interior	-0.043	-0.361	0.278	0.792
	Open	0.022	-0.348	0.391	0.907
Vestiture	Glabrous/pubescent	-0.011	-0.227	0.197	0.930
Leaf size	N/A	-0.014	-0.343	0.328	0.927



