Ant association facilitates the evolution of diet breadth in a lycaenid butterfly

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The role of mutualistic interactions in adaptive diversification has not been thoroughly examined. Lycaenid butterflies provide excellent systems for exploring mutualistic interactions, as more than half of this family is known to use ants as a resource in interactions that range from parasitism to mutualism. We investigate the hypothesis that protection from predators offered to caterpillars by ants might facilitate host-range evolution. Specifically, experiments with the butterfly Lycaeides melissa investigated the role of ant association in the use of a novel host, alfalfa, Medicago sativa, which is a sub-optimal host for larval development. Survival on alfalfa is increased by the presence of ants, thus supporting the hypothesis that interaction with ants might be important for host-range evolution. Using a demographic model to explore ecological conditions associated with host-range expansion in L. melissa, we conclude that the presence of ants might be an essential component for populations persisting on the novel, sub-optimal host.

Keywords: enemy-free space; introduced host plant; niche breadth; niche shift; specialization

1. INTRODUCTION
Parasitic interactions, in particular the relationship between herbivorous insects and their host plants, have featured prominently in theories of adaptive diversification [1–4]. By contrast, the role of mutualistic interactions in evolutionary diversification has been less explored [5,6]. Butterflies in the families Lycaenidae and Riodinidae have been a focal point for interest in the connection between diversification and mutualistic interaction [7–10]. Approximately, one third of all butterfly species are lycaenids, and more than half of all lycaenids are myrmecophilous: they engage in interactions with ants that are either mutualistic, commensalistic or parasitic [11]. A common mutualistic interaction involves secretions provided by caterpillars from specialized glands in exchange for protection provided by ants from natural enemies. Certain facets of these ant–caterpillar interactions have been studied in great detail, such as behavioural and chemical communication between ants and caterpillars [11,12], as well as the ecology of exchanging reward for protection [13–15]. These interactions have also been of interest for their relevance to the idea of enemy-free space [16]. The evolutionary implications have been less thoroughly studied, although one long-standing hypothesis is that these interactions, in which caterpillars benefit from the presence of ants, might influence the diet breadth or host-range evolution of the butterflies. Specifically, an observation derived from surveys of ant–caterpillar associations is that lycaenid butterflies which engage in mutualistic interactions, particularly obligate interactions, have a broader host range than non-myrmecophilous species [17].

A number of mechanisms have been suggested to explain this association between the ant interaction and diet breadth [8], however, most attention has focused on two complementary possibilities: (i) the presence of ants influences oviposition decisions, such that a novel host might be used if ants are available and likely to tend, and thereby protect, offspring; and (ii) the presence of ants creates a sufficient reduction in predator pressure to facilitate survival on novel hosts [7]. The former phenomenon (ant-associated oviposition) has received the most attention [18–21]. Here, we focus on the latter possibility, that ant protection facilitates larval survival on a novel host. To our knowledge, this study represents the first experimental investigation of an ant–caterpillar association in a system involving a recent diet breadth expansion.

We focus on the genus Lycaeides in North America, a complex of species that has been the subject of ecological and evolutionary studies on a diversity of topics including mate choice, host-plant adaptations, Wolbachia infection and hybrid speciation (e.g. [22–25]). Lycaeides melissa [26] is found throughout much of the continent in association with leguminous larval hosts, and notably with both cultivated and feral alfalfa, Medicago sativa, that L. melissa has colonized within the past 200 years [27]. Previous work has suggested that, under certain conditions...
(such as the absence of flowers that larvae will consume), *M. sativa* is a poor larval resource (being associated, at least under laboratory conditions, with reduced survival and smaller adult size; [24]). We focus here on a population of *L. melissa* using two sympatric, larval host plants: a native host, *Astragalus canadensis* and the novel host, *M. sativa*.

The presence of the ant–caterpillar association on both native and novel hosts provided the opportunity to address the potential importance of ant protection for the evolution of lycaenid host range. First, we investigated the community context for our focal ant–caterpillar interactions by asking (i) if there are differences in the richness and abundance of natural enemies associated with the two host plants. We also asked (ii) if ants and insects tended by ants are more or less abundant on the novel host relative to the native host. To address the importance of ants for host use by *L. melissa*, we asked: (iii) what are the consequences of ant association for larval survival in the wild? and (iv) do ants have a similar effect on both the native and exotic hosts? Finally, we incorporate the survival data presented here with previous knowledge of this system into a demographic model that allows us to ask: (v) how might the abundance and the quality of the native host and the abundance of mutualistic ants interact to determine the persistence of *L. melissa* populations on a sub-optimal, novel host?

2. MATERIAL AND METHODS

The study described here was conducted in a sagebrush and riparian area on the western edge of the Great Basin, in Lassen County, California, between Beckwourth Pass and the town of Hallelujah Junction at approximately 1460 m elevation (we refer to our study locality, 39.78° N, 120.07° W, simply as Beckwourth Pass). The *L. melissa* population at Beckwourth Pass has three to four generations per year; these experiments were conducted at the end of July and the beginning of August during the flight of the third generation.

*Lycaenides melissa* caterpillars have been observed at Beckwourth Pass being tended by ants on both hosts (M. L. Forister 2009, personal observations). Ant association is common in later-instar lycaenid caterpillars, as organs associated with ant reward and appeasement typically develop after the second instar [11,28], although instances associated with ant reward and appeasement is common in later-instar lycaenid caterpillars, as organs (M. L. Forister 2009, personal observations). Ant association Beckwourth Pass being tended by ants on both hosts.

In the first experiment, larvae were placed in a blocked, fully crossed design on both plants, with and without the exclusion of ants. Each block consisted of a pair of haphazardly chosen plants (one *M. sativa*, the novel host, and one *A. canadensis*, the native host) that were adjacent or nearly adjacent (in some cases interdigitated, and never more than 3 m apart). On each plant, a pair of adjacent and accessible branches was haphazardly chosen to receive experimental caterpillars, and nearby branches were trimmed when necessary so that foliage blowing in the wind would not cause non-experimental branches to come into contact with focal branches (trimming was necessary on both plants, but kept to a minimum to avoid introducing experimental bias). Insects (including *L. melissa* caterpillars) and spiders were initially removed following visual inspection of all experimental foliage. From each pair of branches, the base of one was haphazardly chosen to be surrounded with Tanglefoot to exclude ants (The Tanglefoot Company, Grand Rapids, MI, USA). Between seven and 10 larvae were then placed on each branch (the number varying over the course of the experiment depending on the availability of larvae); thus each block (two plants and four branches) involved between 28 and 40 caterpillars. The range of caterpillars per individual plant (14–20 caterpillars) falls within the densities of caterpillars that we have observed on plants at this and other *L. melissa* populations, which can be as high as 25 caterpillars per plant (C. Scholl & M. L. Forister 2010, unpublished data).
Caterpillars remained on branches in the field for approximately 6 h, from mid-morning to late afternoon. At the end of that time, each branch was clipped and sealed in a plastic bag. The same evening, branches were thoroughly searched and larvae counted. Experimental exposure of larvae to field conditions was confined to a limited number of hours because observations of ants and caterpillars at Beckwourth Pass had suggested that caterpillars left until dusk would leave their day-time positions, possibly moving off the plants or to other branches (a disappearance that would appear to be a predation event in the context of this experiment).

The second experiment addressing the importance of ants for larval survival in the field focused solely on *M. sativa*, with the aim of increasing our power to investigate the importance of ants for larval survival on the novel host. In this experiment, *M. sativa* plants were again haphazardly located (avoiding plants used in the previous experiment), and eight experimental branches were chosen on each plant. Branches were alternately assigned to the treatment with ants excluded or without ants excluded, with Tanglefoot applied as before. A single larva was then placed at the tip of each branch, on an inflorescence (which is where predators, such as coccinellid beetles, have been observed by us foraging on *M. sativa*). As before, branches were individually bagged at the end of the day, and the presence or absence of larvae scored.

(c) Analyses of caterpillar survival

Data from the first experiment (the crossed design, with both plant species, with and without the exclusion of ants) consisted of the fraction of larvae surviving on each branch. These data violated the assumptions of normality and homogeneity of variance necessary for analysis of variance (ANOVA). As transformations did not correct these issues, we employed a permutational ANOVA (PERMANOVA) in the program PRIMER v. 6, which calculates pseudo-F statistics and significance values based on 1000 permutations of the data, and allows for the analysis of a crossed design in a non-parametric framework [32]. PERMANOVA is not affected by departures from normality, though it can be inappropriate for parametric analysis, and were analysed with the Wilcoxon signed-rank test,computed with the normal approximation (owing to the large number of ties) [31].

(d) Demographic simulations

To explore the role of ant protection in the persistence of *L. melissa* populations, we used a stage-structured demographic model including four life-history stages (egg, larva, pupa and adult), with values for stage-specific survival and fecundity varying stochastically among generations and individuals (full model details in the electronic supplementary material, appendix A). Values for survival and fecundity (as well as other parameters, discussed below) were drawn from rescaled beta distributions (α = 2; β = 3; see the electronic supplementary material, appendix A for more details). In addition to the simple survival and fecundity values, we included ant-tending, variable host availability (the proportion of the host pool composed of *M. sativa*) and variable host flowering. The different hosts have distinct effects on larval growth and survival, and the availability of flowers affects not only larval performance (flowers improve larval performance on *M. sativa*, but have little effect on the native host), but also oviposition [24]. Without flowers, the native host is preferred by ovipositing females; with flowers, plants are chosen with equal frequency [24]. Flower availability can also affect secretions produced by caterpillars [34], although this has not yet been studied in *L. melissa*, and is not included in our models.

We parameterized our demographic simulations with a combination of sources, including our previous work with *L. melissa* [24,35], the work reported here, and other sources as described below. Our goal for demographic modelling was not to generate quantitative, demographic predictions. Rather, our goal was to qualitatively explore population extinction and persistence under different ecological scenarios. Values used for parameters associated with survival in three early life-history stages and egg production (parameters 1–4, table 1) come from detailed studies of the closely related Karner blue butterfly, *Lycaeides melissa samuelis* [25,36,37]. However, the exact values for these parameters are important only insofar as they produce stable populations in our simulations, and thus serve as a baseline for populations on the native host. It is important to note how this framework for demographic simulations interacts with our experimental work: because our field experiments are of short duration and involve experimentally manipulated caterpillars, we do not use our experimental data to generate specific survival values for entire life-history stages. Instead, we use information about relative survival on the two hosts (both from laboratory and field experiments) under different conditions to modify our base survival rates, making the assumption that our performance and survival data are informative in a comparative sense of the larval life-history stage on the two plants.

Beyond the first four parameters, key parameter values used in simulations are derived from work with *L. melissa* at our focal population. For example, from the empirical results described below, we calculated a ratio of survival on *M. sativa* with and without ants, and then used that value to modify larval survival in simulations (*Medicago/AntFactor*, table 1). More details on the derivation of parameter values are provided in the electronic supplementary material, appendix B. Parameters 11–14 (table 1) are our parameters of primary interest, for which we explored a range of values in our simulations. For both the proportion of larvae tended by ants (Tend), and the proportion of *M. sativa* in the host pool (*FreqMedicago*), simulations were run across all combinations of values from 0 to 1, in increments of 0.01. We evaluated a smaller number of parameter values for *AFmin* and *MFmin*. These are the minimum proportion of plants flowering for both species (the maximum proportion of plants flowering was always set at 1). By setting a lower minimum value for flowering, we are increasing the variation (across generations) in the proportion of flowering plants.

For different combinations of our factors of interest (Tend, *FreqMedicago*, *AFmin* and *MFmin*), 1000 replicate simulations were run for 100 generations. This is a relevant time frame for investigating the recent colonization of an exotic host that has been available for less than 200 years [27], and population dynamics were found to be generally stable after 100 generations. Finally, for three life-history
stages associated with values from *L. m. samuelis* (egg-to-larva survival, larva-to-pupa survival and pupa-to-adult survival), we explored a range of parameter values to understand the sensitivity of our results to the baseline values taken from the literature. Demographic simulations were executed in R [38], with code written by the authors, and available upon request.

### 3. RESULTS

#### (a) Host-associated communities

To characterize the plant-associated communities of natural enemies, ants and ant-tended Hemiptera, 605 individuals were identified to family (identifications were made to genus in two cases: ants and a predatory lygaeid bug in the genus *Geocorus*). We found that natural enemies were more abundant on the native host: 2.35 individuals per taxon on *M. sativa* (1.04 s.e.), and 3.8 individuals per taxon on *A. canadensis* (1.27 s.e.; *T* = 2.68, *p* = 0.007). Enemy richness was also higher on the native host: 17 potentially predatory families were found on *A. canadensis*, 10 were found on *M. sativa*. The numerically dominant predators were the same on both plants: *Geocorus* bugs (Lygaeidae) accounted for 29.8 per cent of individuals on *M. sativa* and 30.3 per cent on *A. canadensis*; crab spiders (*Thomisidae*) accounted for 34 per cent of individuals on *M. sativa* and 21 per cent on the native host. In contrast to the greater abundance and the richness of enemies on the native host, the number of ant individuals was higher on the exotic host (a total of 142 individuals compared with 23 individuals on *A. canadensis*), and the number of ant-tended Hemiptera (*Cicadellidae, Membracidae* and *Aphididae*) was also higher on *M. sativa* (115 individuals compared with 54 on *A. canadensis*). The ants collected from the two hosts all belonged to the genus *Formica*, and comprised at least five species: *Formica oreas, Formica rufa*, *Formica rufa*, *Formica neoguineus* and *Formica lasioides* (P. Ward 2009, personal communication). These species are difficult to distinguish, and the present study does not address the distribution of ant species across the two hosts.

#### (b) Caterpillar survival

A total of 338 caterpillars were put on plants in the field during the 3 days of the first experiment (the crossed design, including both plants with and without ants excluded), in a total of 10 blocks. One-hundred and ninety-nine caterpillars survived. A significant effect of plant species was detected on the survival of caterpillars: the average fraction surviving on the native host was 0.74 (+ 0.04 s.e.), while on *M. sativa* survival was 0.82 (+ 0.06 s.e.; figure 1 and table 2). Although some caterpillars might have fallen from plants, in general, caterpillars were observed to be extremely sedentary, in some cases being found in the same position on a leaf at the end of the day that they were placed at the start. Rings of Tanglefoot on ant-exclusion treatments were examined for evidence that caterpillars attempted to crawl from the branches, but no caterpillar was ever found trapped in the Tanglefoot. Thus, we assume that at least a portion of the missing caterpillars represent predation events.
Considering the short duration of the experiments, these are high rates of mortality and predation. The young larvae used in these experiments might have been particularly vulnerable: predation pressure has often been observed to be unequal across larval development [39], with earlier instars in many cases being the most attacked [40]. During the course of the experiment, we frequently observed predators foraging on experimental branches, and in one case, a jumping spider (Salticidae) was seen removing a caterpillar (jumping spiders moved laterally from branch to branch and thus were not prevented by Tanglefoot from accessing branches). We can not be certain that non-experimental larvae did not colonize experimental branches (those without Tanglefoot) during the course of the experiment, which would alter our survival results. This seems unlikely, given the sedentary nature of the early instars and the fact that branches were cleared of insects before the start of the experiment.

Although there was an effect of plant on larval survival (figure 1), we did not detect an effect of ant exclusion on larval survival in this first experiment. However, we did observe an increase in variance in survival on M. sativa in the presence of ants relative to survival associated with the other treatments (Levene’s test for homogeneity of variance across treatments: $F_{3,36} = 4.57, p = 0.0082$). We hypothesized that this could be the result of stochasticity in ant association. The alfalfa plants involved in experiments were large, having approximately 20–40 branches. The experimental larvae were on two of those branches, and ants were excluded from one. Thus, there was perhaps a small chance that ants, even if present on a plant, would find and protect a particular larva. Also, there is the possibility that certain species of ants might have preyed upon larvae, which would have introduced a greater variance in survival under the ant treatment.

As mentioned previously, the heterogeneous nature of the variance across groups (figure 1) can bias distribution-free approaches (like PERMANOVA) just as it can with parametric tests. However, the increased variance associated with the presence of ants on M. sativa does not affect our overall conclusion for this first experiment that predation is higher on the novel host: if we consider, for example, a two-way comparison between survival on the two hosts only under the no-ant treatment, survival is significantly higher on the native host ($T = -2.40, p = 0.014$; Wilcoxon signed-rank test).

Our second experiment, focusing only on M. sativa, included eight branches per plant, with four of those including ants, thus increasing the likelihood for detecting ant–caterpillar interactions. This experiment, involving 80 caterpillars and 10 experimental blocks detected a significant effect of ant presence on larval survival ($T = -2.71, p = 0.006$): in the presence of ants, the average fraction surviving was 0.65 ($\pm 0.06$ s.e.), while without ants survival was 0.28 ($\pm 0.07$ s.e.; figure 2). It is worth noting that average survival across both treatments in this experiment was 0.46 ($\pm 0.27$ s.e.), which is consistent with M. sativa-associated survival values across both treatments (with and without ants) from the first experiment (figure 1).

The benefit that caterpillars derive from the presence of ants might be direct (when ants directly tend and protect an individual caterpillar) or indirect, if the presence of ants on a branch or a plant deters natural enemies from attacking caterpillars even if a particular caterpillar is not tended by ants. The earlier-instars used in experiments have not been observed producing rewards for ants. However, during the course of the second experiment, ants were observed in many cases patrolling branches containing our experimental larvae (ants were never seen on the ant-exclusion branches), and the larvae were often investigated by patrolling ants. Most probably, the ant-associated benefit shown in figure 2 is largely a result of the presence of ants patrolling foliage and tending larger caterpillars and other insects (hemipterans), rather than focused tending and protection of experimental caterpillars. The indirect nature of the protection does not of course mitigate the survival advantage gained by L. melissa caterpillars in the presence of ants (figure 2).

(c) Demographic models

The two parameters of primary interest were the proportion of available hosts that were M. sativa and the proportion of larvae tended by ants. We found that very high levels of available M. sativa result in a population that is dependent on the presence of ants (figure 3a). In particular, if the presence of M. sativa is at or near 1, then populations only persist when ant tending is also high (greater than approx. 0.8). If M. sativa presence is lower, particularly less than 0.6, population persistence becomes insensitive to the presence of ants. The dependence on ants with a high M. sativa presence is a consequence of the extremely low rate of survival that we have discovered for caterpillars on M. sativa without ants. As mentioned above, we detected high rates of mortality in the field, particularly on the novel host. As these mortality values were based on experiments of short duration, the possibility should be considered that our demographic predictions for survival on the novel host are overly dire. To address this, we ran simulations that increased the maximum rate of larva-to-pupa survival to 70 per cent and 90 per cent. Our basic result
Table 2. Details from PERMANOVA analyses of survival data (figure 1). SS, sum of squares; MS, mean square.

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4. DISCUSSION

The importance of mutualisms for community structure and coexistence has been widely appreciated [41–43]. With important exceptions (e.g. [44,45]), mutualistic interactions have received less attention as components of adaptive diversification. This has been recently discussed with respect to mutualistic microbial symbionts and host range in herbivorous insects [6,46]. For lycaenid butterflies, at least two prominent (and complementary) mechanisms have been discussed through which the presence of ant mutualists might interact with host-range evolution: ant-associated oviposition and ant protection [11]. Most previous studies have focused on the former (e.g. [19]), here, we provide support for the latter. We find that the presence of mutualistic ant partners facilitates the use of a novel host by *L. melissa* through the amelioration of predation. The novel host, *M. sativa*, is physiologically such a poor host for *L. melissa* [24], that without that buffering against predation, populations of the butterfly might not be able to persist solely on the novel host (figure 3).

These results are consistent with previous research on conditional mutualisms [47,48], including studies that have focused on associations between ants and treehoppers (Membracidae), which engage in mutualistic interactions that are important under certain community conditions, such as the presence of particular natural enemies, but less so under other conditions [49]. These results also provide an interesting complement to work on predation pressure and enemy-free space in plant–insect interactions [50]. Enemy-free space, in the context of flowers being a poor host [24]. In terms of the model, when the minimum fraction of flowers is lowered, the lowest possible survival on *M. sativa* (represented by *MedicagoQualFactor*) is experienced by a greater fraction of larvae.

The effect of flowering *A. canadensis* is more subtle. While *M. sativa* flowers have an impact on larvae, *A. canadensis* flowers have an impact on adult behaviour. When both hosts are without flowers, *A. canadensis* is the preferred host (the foliage of the native is attractive to females, but not the flowers, which do not provide nectar for adults), but when both hosts have flowers *A. canadensis* and *M. sativa* are equally preferred [24]. The consequence of this is that when more *A. canadensis* plants are flowering, proportionally more eggs are distributed onto *M. sativa* (the lower quality host) compared with when less *A. canadensis* are flowering. This effect can be seen in the comparison between figure 3c and 3d.

Figure 2. Proportional survival of *L. melissa* caterpillars experimentally placed on *M. sativa* branches with and without ants excluded. Each replicate (pair of bars) corresponds to a single *M. sativa* plant, and eight experimental branches (four with ants excluded, and four accessible to ants). In two cases (replicates five and seven), survival in the absence of ants was zero. Black bar, ants; grey bar, no ants.

(dependence on ants in the presence of *M. sativa*) is qualitatively similar under these different regimes of larval survival (electronic supplementary material, appendix C). We also ran simulations that addressed the sensitivity of our results to parameterization of egg-to-larva survival and pupa-to-adult survival. In both cases, a large increase in the range from which survival values were drawn resulted in similar results (electronic supplementary material, appendix C), suggesting that our primary conclusions hold over a wide range of parameter values.

This interaction between *M. sativa* presence and ant tending is modified by the fraction of plants that are flowering. In general, as the minimum fraction of plants flowering was lowered (figure 3b–d), the region of parameter space increased over which populations went extinct. Ecologically, having a low minimum fraction of plants flowering is meant to correspond to a plant population that is less vigorous than a population in which all individuals are always flowering. Variation in the fraction of plants flowering could also reflect temporally variable conditions experienced by different generations within a single population. The effect of lowering the minimum flowering fraction in simulations (figure 3) is primarily a consequence of the fact that *M. sativa* in the absence of

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of host-range ecology, suggests that diet breadth might be affected by novel hosts, which provide an escape from predators associated with ancestral hosts. The Alaskan swallowtail butterfly (*Papilio machaon aliaska*), for example, experiences reduced mortality from predators when using two novel hosts relative to an ancestral host [51].

For *L. melissa*, the novel host does not represent enemy-free space: although predators are less numerous on the novel host, predation rates are in fact higher on *M. sativa* (figure 1). The higher rates of predation on the novel host could be a consequence of differences in architecture between the native and novel hosts. *Astragalus canadensis* has a convoluted structure, with long pinnate leaves that are often heavily interdigitated among branches, possibly providing numerous opportunities for concealed feeding by caterpillars. Caterpillars recovered at the end of experimental trials had in fact in some cases moved to the underside of *A. canadensis* leaves, which was not true of caterpillars on *M. sativa*, which had more often moved to forage on flowers. In contrast to the convoluted architecture of *A. canadensis*, *M. sativa* has a relatively simple structure with branches that are quickly and efficiently searched by predators that start at the tips and move down (M. L. Forister 2009, personal observation). Thus, fewer predators could have a greater impact on *M. sativa* as a consequence of plant traits that remain to be investigated directly.

Alternatively, the differences in predation across hosts could be the result of an unknown experimental artefact of manipulating caterpillars that respond differently to being placed on the two plant species, perhaps falling from *M. sativa* more readily. However, our more important result, with respect to the role of ants in the colonization of the novel host, comes from the experiment dealing with a single host (figure 2). Specifically, the presence of ants provides a space in which enemy pressure is at least ameliorated on *M. sativa* facilitating survival on the novel host. Ants are considerably more abundant on the novel host, as are other species (hemipterans) with which ants also have mutualistic interactions.

Previous work on the colonization of *M. sativa* by *L. melissa* suggested that females were using the novel host in response to the presence of adult resources (nectar), despite the fact that the novel host is, relative to the ancestral host, a poor resource for developing larvae [24]. Just as that study demonstrated the importance of studying multiple facets of a host plant as they interact with insect behaviour, the present study advances our understanding of host-range evolution by including the influence of mutualists and predators. We have yet to study parasitoids, which can be a major source of mortality for caterpillars [52], or the differential protection offered by different ant species [53,54]. Thus, we expect our understanding of host use by *L. melissa* to continue to evolve as we deepen our understanding of the complex web of ecological interactions in which host-range evolution occurs in nature [55].

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