RESEARCH ARTICLE SUMMARY

MICROBIOME

Ecology-relevant bacteria drive the evolution of host antimicrobial peptides in *Drosophila*

M. A. Hanson*, L. Grollmus, B. Lemaitre*

INTRODUCTION: Antimicrobial peptides (AMPs) are host-encoded immune effectors first characterized for their role in fighting infection. AMPs are also important in determining the composition of the host microbiome in both plants and animals. Although many studies have shown rapid evolution of AMPs, little is known about the selective pressures driving that evolution.

RATIONALE: The host microbiome should exert a substantial selective pressure on host immune molecules because the host must maintain a delicate balance with its microbial associates. Variation in a single AMP can upset this balance, as suggested by recent investigations across diverse taxa. In *Drosophila*, previous studies have shown the AMP family *Diptericin* (*Dpt*) evolves rapidly, including a major effect of the amino acid polymorphism S69R of DptA on host defense against the opportunistic pathogen *Providencia rettgeri*, and *Providencia* spp. are commonly found in fly microbiome communities. Beneficial bacteria of the host microbiome also grow out of control in flies lacking multiple AMP gene families, particularly the gut mutualist *Acetobacter*. *Drosophila* species encode two *Diptericin* genes, *DptA* and *DptB*, which are the product of an ancestral duplication stemming from a *DptB*-like gene. To test the idea that the host immune repertoire might be specifically evolved for controlling common microbiome bacteria, we screened recently made *Drosophila* AMP mutants for defense against infection by *Acetobacter* spp. to determine whether any of the AMP genes could explain how flies keep this mutualistic microbe under control.

RESULTS: We found that a single AMP gene, *DptB*, explains the host ability to resist infection by multiple *Acetobacter* species. This interaction is highly specific: We confirmed that *DptA* does not contribute to defense against *Acetobacter*, whereas *DptB* does not contribute to defense against *P. rettgeri*. We therefore determ the evolutionary history of the *Diptericin* locus, and performed a systematic review of microbiome literature of *Drosophila* and other Diptera. We realized that there have been at least two events of convergent evolution toward *DptB*-like genes in flies feeding on fruit, an ecology associated with high levels of *Acetobacter*. These observations suggest that *DptB* evolved to control *Acetobacter* in the fruit-feeding *Drosophila* ancestor. Moreover, flies that secondarily adopted a mushroom-feeding ecology have repeatedly lost their *DptB* genes, alongside an absence of *Acetobacter* in mushroom-breeding sites. A similar pattern of evolution is also seen in flies that have developed a plant-parasitic ecology, which have lost both *DptA* and *DptB* genes and have an ecology lacking both *Providencia* and *Acetobacter*. To investigate whether these AMP-microbe specificities are shared throughout *Drosophila*, we infected species from across the phylogeny with a diverse complement of *DptA*- and *DptB*-like genes and alleles. We included species with a diversity of *DptA*-like genes, and both *Drosophila melanogaster* and mushroom-feeding flies with or without *DptB*. Host resistance to infection by *P. rettgeri* and *Acetobacter* was readily predicted using just *DptA* or *DptB* presence and polymorphism status, even across fly species separated by about 50 million years of evolution.

CONCLUSION: Our study shows how two microbe-specific defences evolved due to an ancestral duplication producing two *Diptericin* genes. We describe a one-sided evolutionary dynamic wherein the host has adapted its immune repertoire to environmental microbes rather than coevolution of host and microbe. This finding helps to explain the evolutionary logic behind the bursts of rapid evolution common in AMP gene families across taxa. Our results also reveal why certain AMPs can have such disproportionate roles in defense against specific microbes: They were evolutionarily selected for that purpose. This realization suggests that the genome can encode "vestigial" immune effectors, AMPs evolved for defense against microbes that are no longer relevant to the host's modern ecology. Thus, derivation and loss of microbe-specific effectors offers the immune system a highly effective mechanism for tailoring host defenses for control of ecologically relevant microbes. ■

Fruit fly experiments demonstrate that the host immune system is uniquely adapted to common environmental microbes. Evolutionary selection can tailor host antimicrobial peptides (chains) to control specific microbiome bacteria. As a defense system common across plants and animals, variations in the repertoire of antimicrobial peptides are likely important as key risk factors for preventing infection by common ecological microbes. [Credit: Diego Galagovsky]

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RESEARCH ARTICLE

MICROBIOME

Ecology-relevant bacteria drive the evolution of host antimicrobial peptides in *Drosophila*

M. A. Hanson[1,2]*, L. Grollmus[1], B. Lemaitre[1]*

Antimicrobial peptides are host-encoded immune effectors that combat pathogens and shape the microbiome in plants and animals. However, little is known about how the host antimicrobial peptide repertoire is adapted to its microbiome. Here, we characterized the function and evolution of the *Diptericin* antimicrobial peptide family of Diptera. Using mutations affecting the two *Diptericins* (*Dpt*) of *Drosophila melanogaster*, we reveal the specific role of *DptA* for the pathogen *Providencia rettgeri* and *DptB* for the gut mutualist *Acetobacter*. The presence of *DptA*- or *DptB*-like genes across Diptera correlates with the presence of *Providencia* and *Acetobacter* in their environment. Moreover, *DptA*- and *DptB*-like sequences predict host resistance against infection by these bacteria across the genus *Drosophila*. Our study explains the evolutionary logic behind the bursts of rapid evolution of an antimicrobial peptide family and reveals how the host immune repertoire adapts to changing microbial environments.

Animals live in the presence of a complex network of microorganisms known as the microbiome. The relationship between host and microbe can vary from mutualist to pathogen, which is often context dependent (*1*). To ensure presence of beneficial microbes and prevent infection by pathogens, animals produce many innate immune effectors as a frontline defense. Chief among these effectors are antimicrobial peptides (AMPs), small, cationic, host defense peptides that combat invading microbes in plants and animals (*2*–*5*). Although many studies have shown important roles for AMPs in regulating the microbiome [reviewed in Bosch and Zasloff (*6*)], presently, we cannot determine why animals have the particular repertoire of AMPs that their genome encodes.

Innate immunity has been characterized extensively in *Drosophila* fruit flies (*7*, *8*). Antimicrobial peptide responses are particularly well characterized in this insect (*2*, *9*, *10*). In *Drosophila*, AMP genes are transcriptionally regulated by the Toll and Imd nuclear factor-κB (NF-κB) signaling pathways (*8*). Recent work has shown that individual effectors can play prominent roles in the defense against specific pathogens (*11*–*19*). Consistent with this, population genetics studies have highlighted genetic variants in AMPs correlated with susceptibility against specific pathogens. A landmark study in *Drosophila* found that a serine-arginine polymorphism at residue 69 in one of the two fruit fly Diptericins, “S69R” of DptA (Fig. 1A), is associated with increased susceptibility to *Providencia rettgeri* bacterial infection (*20*). A loss-of-function study later showed that flies lacking both *Diptericin* genes (“Dpt^{SK1},” flies lacking *DptA* and *DptB*) are as susceptible to *P. rettgeri* infection as Imd pathway mutants, whereas flies collectively lacking five other AMP families nevertheless resist infection in a manner similar to the wild type (*21*). Like these investigations in *Drosophila*, a G49E polymorphism in the AMP Calprotectin of Persian domestic cats is associated with susceptibility to severe ringworm fungal skin disease (*22*). Similar AMP variation is common across animals (*23*–*26*). However, although *P. rettgeri* is an opportunistic pathogen of wild flies and ringworm is common in certain cat breeds, whether these AMPs are evolving to selection imposed by these microbes is unclear. Given recent studies on AMP roles beyond infection (*27*–*31*), other fitness trade-offs could also explain AMP evolution.

It is now clear that antimicrobial peptides shape the microbiome (*6*), but defining if or how the host immune repertoire itself is shaped by the microbiome has been challenging. Here, we characterized the function and evolution of the *Diptericin* gene family of flies, revealing that these AMPs were selected to control ecologically relevant microbes.

Results

Diptericin B *is specifically required for defense against* Acetobacter *bacteria*

Acetobacter bacteria are mutualists of *Drosophila* that supplement host nutrition and are common in wild flies (*32*–*35*). We previously showed that a strain of *Acetobacter* grows out of control in the gut of *Relish* mutant flies (Rel^{E20}) lacking Imd pathway activity and in flies carrying deletions removing 14 AMP genes (*ΔAMP14*) (*36*). Here, we identified this *Acetobacter* species as *A. sicerae strain BELCH* (fig. S5). Gnotobiotic association with *A. sicerae* did not cause mortality, even in *ΔAMP14* flies (fig. S6A). However, pricking flies with a needle contaminated with *A. sicerae* killed *ΔAMP14* flies (*12*, *36*), also causing an abdominal bloating phenotype that preceded mortality (shown later). This route of bacterial infection is similar to what flies experience when their cuticle is pierced by natural enemies [e.g., nematodes, wasps, and mites (*37*–*39*)]. Because *ΔAMP14* flies are killed by *A. sicerae* systemic infection, one or more AMPs are likely required to control opportunistic infections by this microbe. We therefore used flies carrying overlapping sets of AMP mutations (*21*), including a *Diptericin* mutant panel affecting each of the two *Diptericins* (Fig. 1B), to narrow down which AMP(s) protects the fly against *A. sicerae* infection.

Ultimately, deleting just *DptB* fully recapitulates the susceptibility of *ΔAMP14* flies. Dpt^{SK1}, $DptB^{KO}$, and $DptB^{A3}$ flies suffered 100% mortality after infection, with survival curves mirroring *ΔAMP14* and Rel^{E20} flies; these *DptB*-deficient flies also presented similar levels of abdominal bloating (Fig. 2, A and B). Furthermore, ubiquitous RNA interference (RNAi) silencing of *DptB* caused both mortality and bloating after *A. sicerae* pricking (fig. S6, B and C). Conversely, $DptA^{S69R}$, $DptA^{\Delta 822}$, and even *ΔAMP8* flies collectively lacking five other AMP gene families [*Drosocin*, *Attacin*, *Defensin*, *Metchnikowin*, and *Drosomycin* (*21*)] resisted infection in a manner comparable to wild type. Finally, *DptB* mutants display increased *A. sicerae* loads, preempting mortality (Fig. 2C), suggesting a direct role for *DptB* in suppressing *A. sicerae* growth.

After revealing the critical importance of *DptB* in defense against *A. sicerae*, we investigated whether *DptB* has a broader role in the control of other *Acetobacter* species. To this end, we infected flies with a panel of *Acetobacter* species including *A. aceti*, *A. indonesiensis*, *A. orientalis*, *A. tropicalis*, and *A. pomorum*. Although these *Acetobacter* species displayed different levels of virulence, *DptB* specifically promoted survival and/or prevented bloating against all virulent *Acetobacter* species (figs. S7 and S8).

Collectively, these results indicate that *DptB* is an AMP of specific importance in defense against multiple *Acetobacter* species, revealing another example of high specificity between an innate immune effector and a microbe relevant to host ecology. Because *Acetobacter* are common in fermenting fruits (*40*, *41*), the major ecological niche of *Drosophila*, *DptB* might be especially important for flies to colonize this niche.

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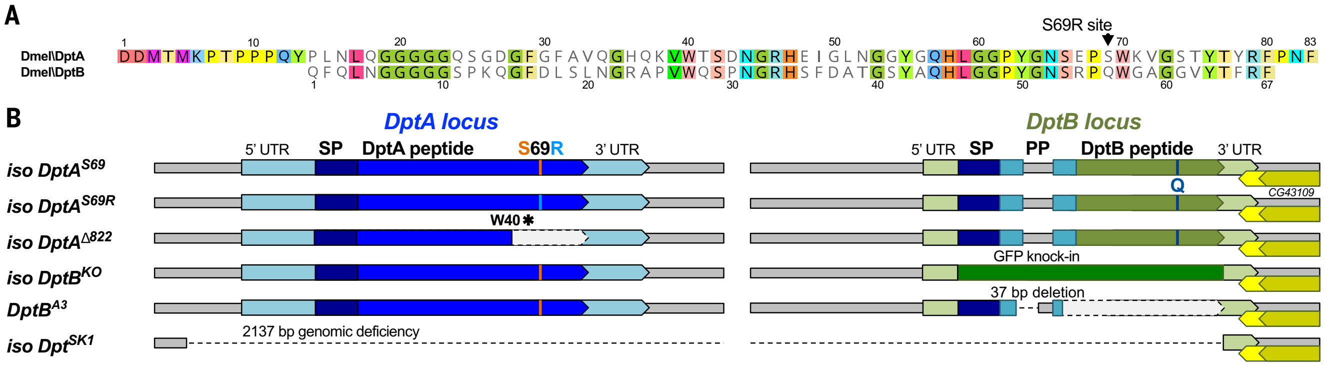


Fig. 1. *Diptericins of D. melanogaster.* (**A**) Alignment of *D. melanogaster* mature DptA and DptB peptides, which are ~52% identical. The DptA^{S69R} site is noted (Q in DptB, and see fig. S1 for protein folding predictions). (**B**) The two *Diptericin* genes are located in tandem on chromosome 2R;55F with only 1130 base pairs (bp) between them. *DptA*$^{\Delta 822}$ encodes a premature stop (W40*). Strain *DptB*^{A3} encodes a 37-bp deletion overlapping the *DptB* intron-exon boundary, causing loss of function (fig. S2). The *Dpt*^{SK1} deficiency removes 2137 bp, deleting the coding region of both genes. *DptB* also encodes a secreted propeptide (PP), similar to *Drosophila* Attacins (figs. S1, S3, and S4). SP, signal peptide.

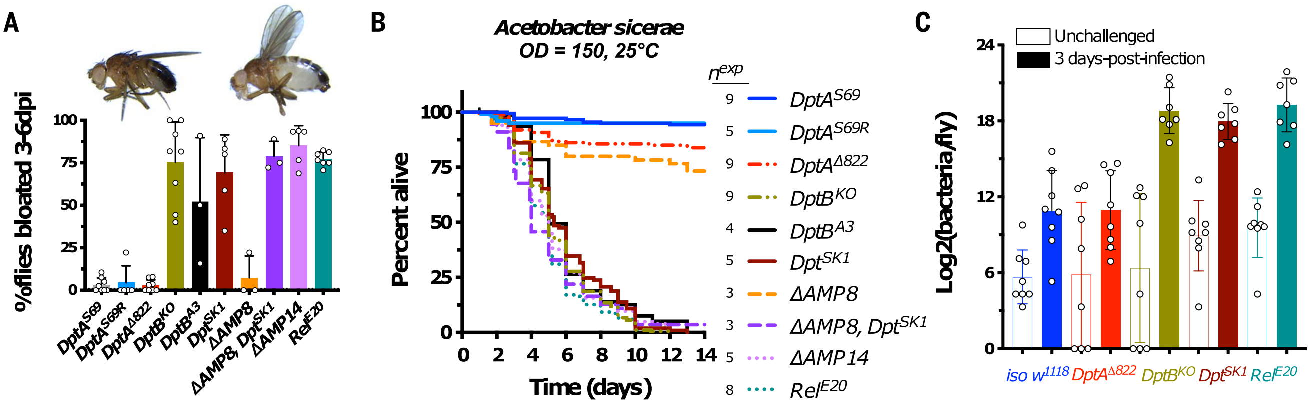


Fig. 2. *DptB is specifically required for defense against A. sicerae.* (**A**) Flies lacking *DptB* bloat after *A. sicerae* systemic infection. Each data point reflects the average from one replicate experiment (~20 males). (**B**) Sum survival curves showing that *DptB* is critical for defense against *A. sicerae*. (**C**) *A. sicerae* bacterial load increases before mortality. Each data point reflects the average of five pooled flies. n^{exp}, number of experiments.

Diptericin A *is specifically required to defend against* P. rettgeri

The Gram-negative bacterium *P. rettgeri* was isolated from the hemolymph of wild-caught flies (20), suggesting that it is an opportunistic pathogen in *Drosophila*. Previous studies showed that *Diptericins* play a major role in surviving *P. rettgeri* infection (20, 21), including a marked correlation between the DptA S69R polymorphism and resistance against this bacterium: Flies encoding arginine were more susceptible than flies encoding serine at this site (20). However, it is unknown if *DptB* contributes to defense against *P. rettgeri*.

We therefore infected our panel of *Diptericin* mutants by pricking with *P. rettgeri* (Fig. 3A). We confirmed the *DptA*^{S69R} allele reduces survival after *P. rettgeri* infection, here with a controlled genetic background ($P < 2 \times 10^{-16}$). *DptA*$^{\Delta 822}$ flies also paralleled mortality of *Dpt*^{SK1} flies lacking both *Diptericin* genes ($P = 0.383$). Initially, we found that *DptB*^{KO} flies showed higher susceptibility to *P. rettgeri* ($P = 9.44 \times 10^{-11}$), correlated with higher bacterial load (fig. S9A). However, our isogenic *DptB*^{KO} flies had only ~57% induction of the *DptA* gene compared with our isogenic *DptA*^{S69} wild type at 7 hours after infection (fig. S9B). By contrast, we observed that *DptB*^{A3} flies carry the *DptA*^{S69} allele, have wild-type *DptA* expression (fig. S2), and actually survive infection by *P. rettgeri* even better than *DptA*^{S69} ($P = 5.03 \times 10^{-4}$; Fig. 3A). Moreover, silencing *DptB* by RNAi did not significantly affect survival against *P. rettgeri* ($P > 0.05$; Fig. 3B). We therefore conclude that *DptB* itself does not have a major effect on resistance to *P. rettgeri*, although a cis-genetic background effect found in *DptB*^{KO} flies causes lesser induction of *DptA* and, accordingly, higher susceptibility.

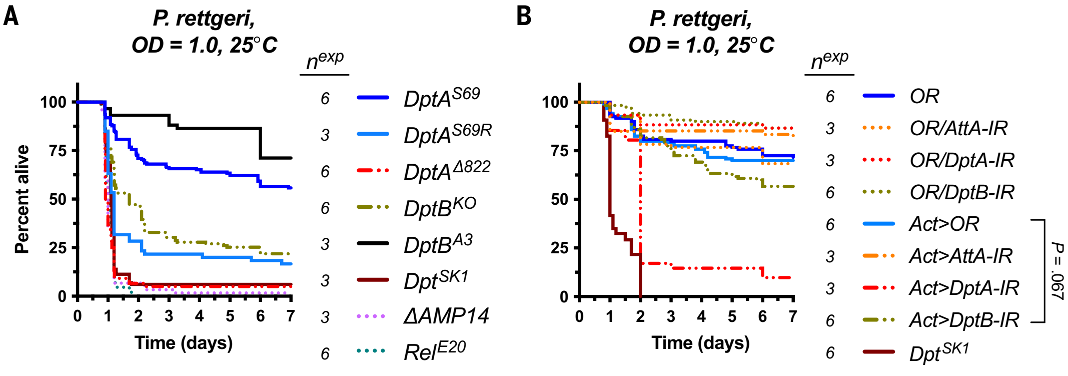
Our *Diptericin* mutant panel shows that *DptA* plays a major role in defense against *P. rettgeri* but not *A. sicerae*. Conversely, *DptB* plays a major role against *A. sicerae* but not *P. rettgeri*. Thus, these two *Diptericin* genes are highly specific effectors explaining most of the Imd-mediated defense of *D. melanogaster* against systemic infection by either bacterium.

The Diptericin *family shows multiple bursts of rapid evolution across Diptera*

Given the high specificity of *D. melanogaster Diptericins* for different ecologically relevant microbes, we next investigated whether host ecology might explain *Diptericin* evolution. First, we reviewed the evolutionary history of *Diptericins* across Diptera using newly available genomic resources (Fig. 4).

Diptericins are found across brachyceran fly species, indicating an ancient origin of this antibacterial peptide (>150 million years ago) (42, 43). The extant *Drosophila DptB*-like gene was originally derived in the Drosophilidae ancestor through rapid evolution [figs. S11 and S12; first shown in (43, 44)]. Later, a duplication of *DptB* gave rise to the *DptA* locus in the Drosophilinae ancestor ~50 million years ago [date per (45)], which began as a *DptB*-like

Fig. 3. *DptA* is specifically required for defense against *P. rettgeri*. (**A**) Sum survival curves of *Diptericin* mutants after infection with *P. rettgeri*. (**B**) Silencing *DptB* by RNAi (*Act>DptB-IR*) does not significantly affect fly survival compared with *Act>OR* controls (RNAi validation is shown in fig. S10).



gene but then evolved rapidly after the duplication [shown in (*44*); also see figs. S11 and S12 and table S1]. Given these repeated bursts of evolution and only ~52% similarity between *DptA* and *DptB* (Fig. 1A), distinct antibacterial activities are not necessarily surprising. In reviewing *Diptericin* evolution, we further realized the $DptA^{S69}$ residue of *D. melanogaster* is also present in the subgenus *Drosophila* through convergent evolution: Different codons are used by the subgenus *Sophophora* (e.g., AGC) and subgenus *Drosophila* (e.g., TCA) to produce $DptA^{S69}$ residues (table S1), providing further evidence that adaptive evolution selects for serine at this site [complementing (*20*) and (*44*)]. Moreover, across species, there is a high level of variation at this site: In addition to the S69R polymorphism, this site can also encode either glutamine (Q) or asparagine (N) in DptA of other *Drosophila* species. Q/N is also seen at the aligned residue of DptB across *Drosophila* species (Q56N in *DptB*). These four residues (S, R, Q, and N) are derived compared with the ancestral aspartic acid residue (D) found in most other dipterans (table S1).

This analysis suggests that the extant *DptB*-like gene first evolved in the drosophilid ancestor, whereas *DptA* emerged from a duplication of a *DptB*-like gene, followed by rapid diversification. The $DptA^{S69}$ residue was also derived at least twice, and this site is highly polymorphic across genes and species. These repeated bursts of evolution suggest that fly *Diptericins* evolved responding to selection in the drosophilid ancestor.

Diptericin *evolution correlates with microbe presence in host ecology*

The diversity of *Drosophila* ecologies, along with many wild-caught fly microbiome studies, places us in a unique position to pair each host's microbial ecology with patterns in the evolution of their *Diptericins*, which have microbe-specific importance.

We performed a systematic review of the Diptera microbiome literature (table S2). *Acetobacter* bacteria are regularly found across species feeding on rotting fruits in microbiome studies (*32*, *34*, *46*, *47*). However, *Acetobacter* appear to be absent from rotting mushrooms (*48*), and are largely absent in wild-caught mushroom-feeding flies themselves (*48*, *49*). Further, *Providencia* bacteria related to *P. rettgeri* are common in species feeding on both rotting fruits and mushrooms [(*34*) and table S2]. We observed that three drosophilid species with mushroom feeding ecology, *D. testacea*, *D. guttifera*, and *Leucophenga varia*, have independently lost their *DptB* genes (Fig. 4) (*43*). Thus, three independent *DptB* loss events have occurred in flies with a mushroom-feeding ecology specifically lacking in *Acetobacter*.

There is another *Drosophila* sublineage with an ecology that lacks *Acetobacter*: *Scaptomyza* (Fig. 4, green branch). *Scaptomyza pallida* feeds on decaying leaf matter and mushrooms, whereas *Scaptomyza flava* and *Scaptomyza graminum* feed on living plant tissue as leaf-mining parasites (*50*). The *S. flava* microbiome shows little prevalence of either *Acetobacter* or *Providencia* (*51*). We investigated whether these *Scaptomyza* species had pseudogenized either of their copies of *DptA* (two genes, *DptA1* and *DptA2*) or *DptB* (one gene). We found independent premature stop codons in *DptA1* in the leaf-mining species *S. flava* (Q43*) and *S. graminum* (G85*), but not in the mushroom-feeding *S. pallida* (fig. S13). We also analyzed the promoter regions of these *DptA* genes for the presence of Relish NF-κB transcription factor binding sites ["Rel-κB" sites from (*52*); fig. S13A], confirming that the *S. pallida DptA1* promoter retains Rel-κB sites and likely immune induction. Thus, *Scaptomyza DptA1* genes show pseudogenization specifically in the leaf-mining species that lack *Providencia* in their present-day ecology. However, *DptA1* appears functional in *S. pallida*, a mushroom-feeding species likely exposed to *Providencia* through its ecology. *Scaptomyza DptA2* genes show variable presence of Rel-κB sites, but no obvious loss-of-function mutations in coding sequence, and *DptA2* remains expressed in *S. flava* (fig. S13B). Screening the *DptB* genes of *Scaptomyza*, we found no obvious loss-of-function mutations in coding sequences. However, all three *Scaptomyza* species lack Rel-κB sites in their *DptB* promoter regions (fig. S13A). Whether due to plant feeding or mushroom feeding, none of these *Scaptomyza* have an ecology associated with *Acetobacter*. Using RNA-sequencing data from the *S. flava* midgut (*53*), we confirmed a lack of expression of both the pseudogene *DptA1* and *DptB* compared with the abundant expression of *DptA2* (fig. S13B). We conclude that *Scaptomyza* species have independently pseudogenized *DptA* and *DptB* genes correlated with presence or absence of *Providencia* or *Acetobacter* in their ecology.

Finally, convergent evolution toward *DptB*-like sequence has occurred in another lineage of "fruit flies": Tephritidae (*43*, *44*) (see figs. S11 and S12 for protein alignment and paraphyly of tephritid *Diptericins* clustering with drosophilid DptB). This family of Diptera is distantly related to Drosophilidae (last common ancestor ~111 million years ago). Like *Drosophila*, many tephritid lineages (e.g., Trypetinae and Dacinae) feed on fruits, but like *Scaptomyza*, one lineage, Tephritinae, parasitizes live plants (Fig. 4, purple branches). In light of the present study, it would seem that the tephritid species that feed on *Acetobacter*-associated fruit (*40*, *54*, *55*) have convergently evolved a *DptB*-like gene, including a parallel Q/N trans-species polymorphism at the critical Diptericin residue (table S1). Like *Scaptomyza*, plant-parasitizing tephritids lack both *Acetobacter* and *Providencia* in their microbiomes (*43*) and have lost their *Diptericin* genes (Fig. 4) (*43*). Thus, *DptB*-like genes evolved in both Tephritidae and Drosophilidae species associated with a fruit-feeding ecology in which *Acetobacter* is a dominant member of the microbiome. The fact that *DptB*-like genes are not found in species unless their ancestor had a fruit-feeding ecology suggests two things: (i) that the *Acetobacter*-rich fruit-feeding niche was colonized before the derivation of *DptB*-like sequence and (ii) that selection imposed by *Acetobacter* resulted in the ancestors of both Tephritidae and Drosophilidae evolving *DptB*-like genes to help control this microbe.

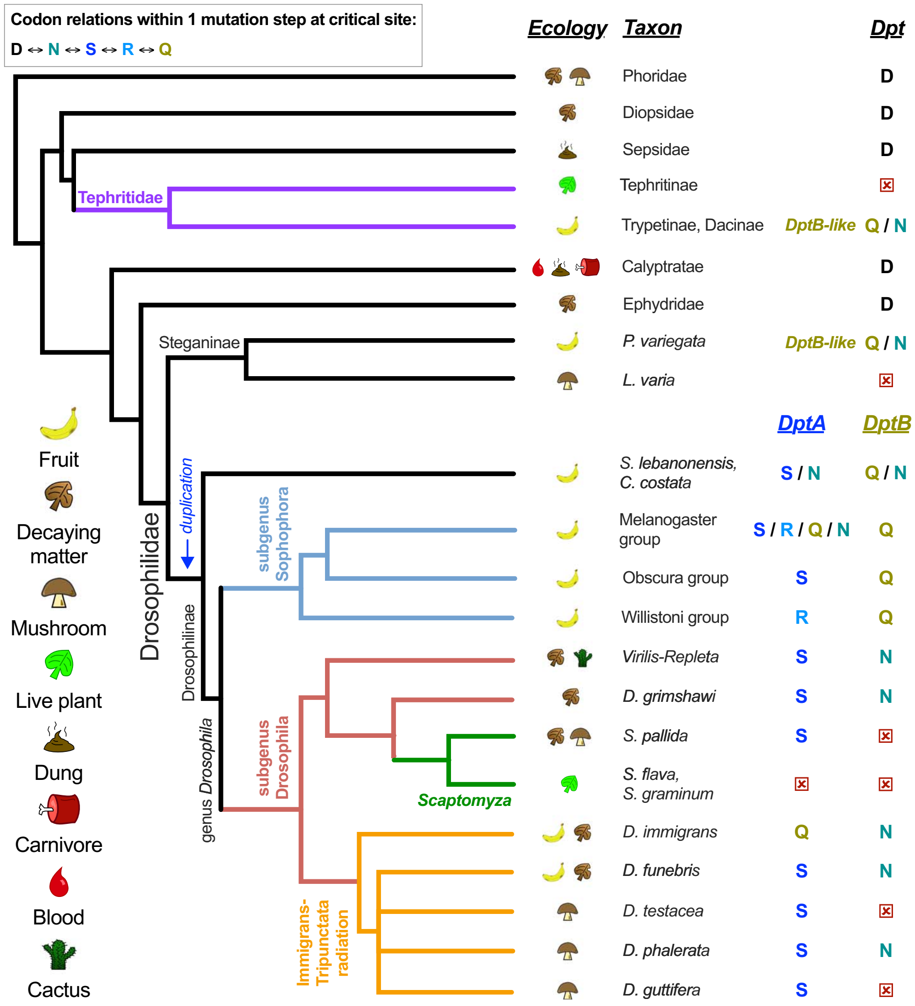


Fig. 4. *Diptericin* evolution correlates with host ecology and presence of *Acetobacter* or *Providencia*. *Diptericin* presence was screened in diverse Diptera. The residue aligned to the DptA^{S69R} or DptB^{Q56N} polymorphism is shown. The *DptB*-like sequence evolved first in the ancestor of Drosophilidae, and the serine-coding allele in *DptA* evolved at least twice (fig. S11 and table S1). The relatedness of the codons used to encode the S/R/Q/N polymorphism enables their diversification in the subgenus *Sophophora* (summary in top left). Fruit-feeding tephritids convergently evolved a *DptB*-like gene (figs. S11 and S12) including a parallel Q/N polymorphism, and *P. variegata* encodes an independent *DptB* duplication, in which the two daughter genes encode either version of the Q/N polymorphism. Within Drosophilidae (bottom part), three species with mushroom-feeding ecology have lost their *DptB* genes: *L. varia*, *D. testacea*, and *D. guttifera*. In both Drosophilinae (*Scaptomyza*) and Tephritidae (Tephritinae), divergence to plant feeding is also correlated with loss of *Diptericin* genes (fig. S13). Systematic review of microbiome studies (table S2) suggests that the absence of *Providencia* and *Acetobacter* in the host ecology is correlated with *DptA* and *DptB* loss, respectively. Red [x] indicates that the gene loss was confirmed. Copy number variation is noted in table S1. Phylogenetic cladogram was drawn from consensus of multiple studies (45, 65–67).

Our phylogenetic and ecological survey reveals multiple parallels among the host immune effector repertoire, ecology, and the associated microbiome. This suggests that these dipteran species have derived *DptA*- or *DptB*-like genes as their evolutionary solution to control important bacteria found in their microbiome. By contrast, specific *Diptericin* genes become superfluous when their hosts shift to ecologies lacking *Diptericin*-relevant microbes, leading to gene loss.

Variation in DptA *or* DptB *predicts host resistance across species separated by 50 million years of evolution*

Our study indicates that among the suite of immune genes involved in *Drosophila* host defense, the AMPs *DptA* and *DptB* are critically important against two environmentally relevant bacteria: the opportunistic pathogen *P. rettgeri* and the gut mutualist *Acetobacter*. Moreover, our phylogeny-microbiome analysis reveals substantial correlations in terms of gene emergence, retention, and loss. If *DptA* and *DptB* really evolved to control *P. rettgeri* and *Acetobacter*, then the outcomes of *P. rettgeri* and *A. sicerae* infection across species should be readily predicted using just variation in these two *Diptericins*. We therefore chose 12 *Drosophila* species with variation in the polymorphic site in *DptA* and presence or absence of *DptB*, and infected them with *P. rettgeri* or *A. sicerae*. Experiments in *D. melanogaster* suggest that *DptA*^{S69R} affects defense against *P. rettgeri*, but how *DptA*^{S69Q} or *DptA*^{S69N} affects defense against this bacterium has never been tested. Similarly, the effect of *DptB*^{Q56N} on defense is also untested, so we have no a priori expectations for how these polymorphisms affect peptide activity. To analyze these experiments, we used a linear mixed-model approach (see the materials and methods), including *D. melanogaster* flies from our *Diptericin* mutant panel as experimental controls. This helped to calibrate our model for the expected effect size for variants of *DptA* or *DptB* within a single species or across species. We also conducted these experiments at 21°C to avoid heat stress to some species, which reduced *D. melanogaster* mortality compared with 25°C (fig. S14).

Summaries of fly species mortality are shown in Fig. 5. As found in *D. melanogaster*, resistance to *P. rettgeri* was associated with a *DptA*^{S69} allele across species. Indeed, *DptA*^{S69R} found in either *D. melanogaster* or *D. willistoni* correlates with increased susceptibility to *P. rettgeri* ($t = -9.59$, $P < 2 \times 10^{-16}$). *Drosophila yakuba* with *DptA*^{S69N} was also more susceptible than its close relatives, suggesting that asparagine (N) is an immune-poor allele against *P. rettgeri* ($t = -7.26$, $P = 4 \times 10^{-13}$). Further, *DptA*^{S69Q} flies (*D. suzukii* and *D. immigrans*) had similar survival after *P. rettgeri* infection compared with *DptA*^{S69} flies ($t = +0.07$, $P = 0.35$), suggesting that glutamine (Q) is a competent defense allele against *P. rettgeri* when coded by *DptA* (Fig. 5A). Overall, ~74% of variation in susceptibility can be attributed to variation in *DptA* alone as a fixed effect (marginal $R^2 = 0.743$).

For infections with *A. sicerae*, the absence of *DptB* in the mushroom-feeding species *D. testacea* and *D. guttifera* was correlated with increased susceptibility compared with their close relatives ($t = -10.83$, $P < 2 \times 10^{-16}$). Mushroom-feeding flies displayed increased susceptibility to *A. sicerae* infection that was independent of *DptB* status ($t = -3.77$, $P = 2 \times 10^{-4}$). However, even within this susceptible lineage, *DptB* loss still increased mortality to a similar extent as *DptB* deletion in *D. melanogaster*, indicating that the contribution of *DptB* to defense against *A. sicerae* is independent of host genetic background (Fig. 5B). Overall, ~87% of variation in susceptibility to *A. sicerae* can be explained by just *DptB*

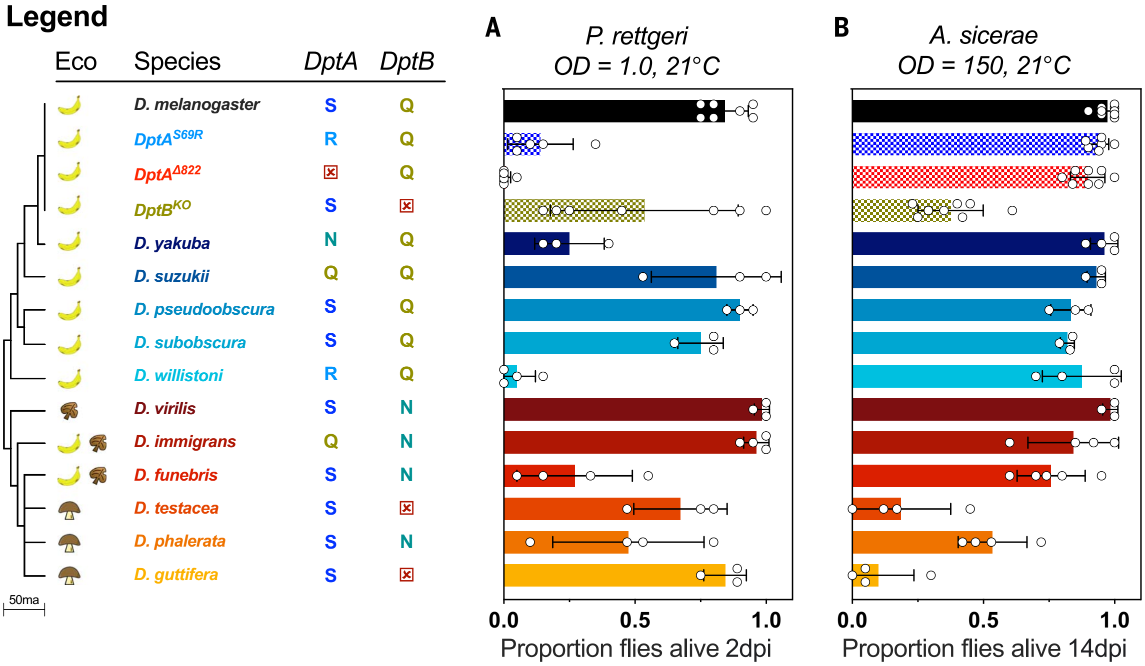


Fig. 5. *Diptericins* predict pathogen-specific survival across *Drosophila*. Host phylogeny, ecology, and *Diptericin* complement are shown. Clean injury is shown in fig. S15. (**A**) Susceptibility to *P. rettgeri* infection varies across species, with survival largely explained by the *DptA* allele, particularly within the subgenus *Sophophora* (blue-shaded species). (**B**) Susceptibility to infection by *A. sicerae* is predicted by presence or absence of *DptB*, although mushroom-feeding flies also had a higher susceptibility to *A. sicerae* infection that was independent of *DptB* loss. Each data point represents one replicate experiment using 20 male flies.

absence and host ecology as fixed effects (marginal R^2 = 0.868).

These survival data establish that the specific resistance conferred by *Diptericins* observed in *D. melanogaster* applies across *Drosophila* species separated by ~50 million years of evolution. We conclude that the host immune repertoire adapts to the presence of ecologically relevant microbes through the evolution of specialized AMPs as weapons to combat specific microbes.

Discussion

Susceptibility to infection often correlates with host phylogeny (*56*, *57*), although host ecology greatly influences microbiome community structure (*34*, *58*). Early studies of immune evolution suggested that AMPs were mostly generalist peptides with redundant function, suggesting that AMP variation was not caused by adaptive evolution (*2*, *3*). Instead, studies on immune adaptation have found whole pathway–level effects or have identified factors specific to a given species [e.g., host-symbiont coevolution (*59*–*61*)]. As a result, despite a rich literature on immunity-microbiome interactions, the evolutionary logic explaining why the host genome encodes its particular immune effector repertoire has been difficult to approach experimentally.

Here, we identified how ecological microbes promote the rapid evolution of effectors of the immune repertoire, tailoring them to be highly microbe specific. The two *D. melanogaster* *Diptericin* genes also provide a textbook example of how gene duplication can promote immune novelty, equipping the host with extra copies of immune tools that can be adapted to specific pathogen pressures. The *Drosophila* Diptericin mechanism of action has been elusive because of technical difficulties in peptide purification (*2*, *10*). However studies using *Phormia terranovae* highlight many directions for future research [(*42*, *62*, *63*) and see discussion in the supplementary materials]. Future studies combining both fly and microbe genetics should be fruitful in learning how host and microbe factors determine specificity. One goal of infection biology is to try to identify risk factors for susceptibility present in individuals and populations. Our study suggests that characterizing the function of single effectors, interpreted through an evolution-microbe-ecology framework, can help to explain how and why variation after infection occurs within and between species.

The fly *Diptericin* repertoire reflects the presence of relevant microbes in that species' ecology. Conversely, loss or pseudogenization of *Diptericins* is observed when the microbes they target are no longer present in their environment. In a sense, this means that some AMPs seen in the genomes of these animals are vestigial: Immune genes evolved to fight microbes that the extant host rarely encounters (e.g., *DptB* in *D. phalerata*). Indeed, flies that lack *DptB* genes are likely disadvantaged on *Acetobacter*-rich food resources, where the possibility of *Acetobacter* systemic infection poses a constant threat. Thus, loss of this AMP makes recolonization of *Acetobacter*-rich rotting fruits a risky proposition, entrenching the host in its derived ecological niche.

Although other mechanisms of defense surely contribute to resistance, *Diptericins* have evolved recurrently as the fly genome's solution to control specific bacteria. Given our findings, we propose a model of AMP-microbiome evolution that includes gene duplication, sequence convergence, and gene loss, informed by the host ecology and the associated microbiome (Fig. 6). In doing so, we thus explain one part of why various species have the particular repertoire of AMPs that they do. This ecology-focused model of AMP-microbiome evolution provides a framework for understanding how host immune systems rapidly adapt to the suite of microbes associated with a new ecological niche. These findings are likely of broad relevance to immune evolution in other animals.

Methods summary

Full materials and methods are found in the supplementary materials. In brief, *D. melanogaster* fly stocks included both natural mutations and a transgenic insertion disrupting *DptB*, which were isogenized into the DrosDel isogenic background, as indicated in Fig. 1 with the prefix "*iso*." Nonisogenic *DSPR A3* flies ($DptB^{A3}$) were from (*64*). Survival experiments were performed and analyzed as described

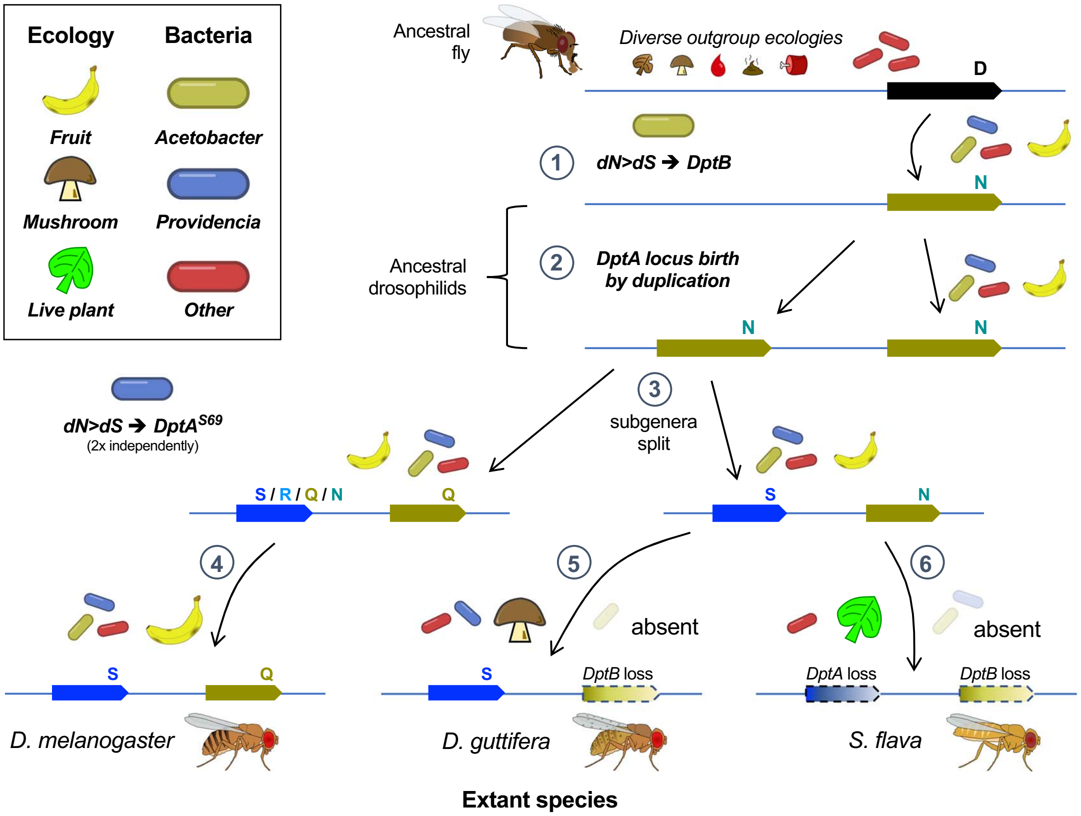


Fig. 6. AMP evolution explained using *Diptericins* and a microbial ecology framework. Outgroup ecologies per Fig. 4. (**1**) The drosophilid ancestor fed on fruit and was consequently exposed to *Acetobacter* bacteria. The *DptB*-like sequence evolved rapidly (dN > dS) to control this new microbe. (**2**) A duplication of *DptB* gave rise to the *DptA* locus. (**3**) The addition of a second *Diptericin* gene permitted evolutionary tinkering to control another relevant microbe, *P. rettgeri*, including convergent evolution of the critical S69 residue. In the sublineage including *D. melanogaster*, codon volatility enables any of S, R, Q, or N residues. The sublineage including *D. guttifera* and *S. flava* evolved its S residue using a different codon, evolutionarily fixing this residue (table S1). (**4**) In *D. melanogaster*, host ecology remains associated with both *Acetobacter* and *Providencia*, which continually select for maintenance of both genes. (**5**) In mushroom-feeding *D. guttifera*, *Providencia* remains a threat, but mushroom ecology lacks *Acetobacter*. Consequently, selection is relaxed on *DptB*, leading to pseudogenization. (**6**) In leaf-mining species such as *S. flava*, *Acetobacter* and *Providencia* are absent from the microbiome, and thus selection is relaxed on both *Diptericin* genes. This AMP-evolution-ecology framework makes sense of why AMPs have microbe specificity and helps to explain how shifts in microbial ecology can promote rapid evolution for AMP-microbe specificity or loss of "vestigial AMPs" that are relevant primarily against microbes that the host no longer encounters.

previously (*21*), with the temperature and optical density at 600 nm (OD_{600}) of the bacteria ("OD") indicated within figures. Twenty male flies were used per experiment unless otherwise indicated, and at least three replicate experiments were performed for all data shown in main figures, with raw data available in the supplement. In fly bloating, bacterial load, and gene expression graphs, error bars indicate SD. The cladogram and annotations in Fig. 4 were generated by literature review (table S2), with gene search and annotation methods per (*43*).

The script for Fig. 5 is available in the supplement. Briefly, we used a linear mixed-model ("lme4" and "performance" packages in R) with species relatedness and experiment block included as random factors and host ecology and variation in *DptA* or *DptB* loci including copy number or alleles at key residues (*D. melanogaster* DptA N52 or S69 alleles) as fixed factors. When loss of function was present, the allele was called as "deleted." We explored our model both by Akaike information criterion model selection and by iterative linear mixed-model testing in which nonsignificant fixed factors (e.g., *DptB* allele in explaining survival after *P. rettgeri* infection) and their interactions were relegated to being random factors in the final model. These two approaches provided similar results, and we used values from linear mixed models in the main text.

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SUPPLEMENTARY MATERIALS

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MDAR Reproducibility Checklist

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Ecology-relevant bacteria drive the evolution of host antimicrobial peptides in *Drosophila*

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Editor's summary

To prevent gut microbiota from running amok, animals and plants secrete a series of small, often multifunctional peptides called antimicrobial peptides. Until recently, antimicrobial peptides were considered to have broad activities, and it was unclear why such molecules showed signs of rapid evolution. Hanson *et al*. found a striking specificity for the peptides diptericin A and B for two species of gut commensal bacteria. These species occur in the natural environment of fruit flies depending on the food resource exploited: fruit or fungi. Thus, the presence or absence of diptericin A or B predicts the ecology of the fly. This work shows how an organism's microbiota might be able to shape the host's immune responses in a manner similar to how a host's immune responses shape its microbiota. —Caroline Ash

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