Mutations in the *neverland* gene turned *Drosophila pachea* into an obligate specialist species

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Abstract

Most living species exploit a limited range of resources. However, little is known on how tight links build up during evolution between specialist species and the hosts they utilize. We examined the dependence of *Drosophila pachea* on its single host, the senita cactus. Several amino acid changes in the Neverland oxygenase rendered *D. pachea* unable to transform cholesterol into 7-dehydrocholesterol (first reaction in the steroid hormone biosynthetic pathway in insects) and thus made *D. pachea* dependent on the uncommon sterols of its host plant. The *neverland* mutations increase survival on the cactus unusual sterols and are in a genomic region that faced recent positive selection. This study illustrates how relatively few genetic changes in a single gene may restrict the ecological niche of a species.

Losses of enzymatic activities are frequent during evolution (1). For example, humans lost the ability to produce nine amino acids and six vitamins, for which we rely on our diet (2). The reasons for such losses are unknown, but it is generally believed that "superfluous" metabolic activities were lost by chance during evolution (3). We examined the dependence of the fly *Drosophila pachea* on the senita cactus (*Lophocereus schottii*), a plant species endemic to the Sonoran desert (Northwestern Mexico and Southwestern USA). In insect developmental transitions and egg production are regulated by the steroid hormone ecdysone (4). *D. pachea* has lost the first metabolic reaction in the ecdysone biosynthetic pathway, i.e. the ability to convert cholesterol into 7-dehydrocholesterol (7DHC) (Fig. 1A, 5–7). The senita cactus, which *D. pachea* requires as a host (5), does not contain common sterols and is the only plant in the Sonoran desert (7) known to produce Δ7-sterols such as lathosterol (6). *D. pachea* flies do not reach the adult stage if not raised on senita cactus, but supplementing standard food with senita cactus or with 7DHC fully restores *D. pachea* viability and fertility (5), indicating that Δ7-sterols are essential compounds required for *D. pachea* development and survival. Interestingly, *D. pachea* appears to depend on the senita cactus solely for its sterols as we raised *D. pachea* on an artificial diet supplemented with 7DHC for more than four years (~60 generations) with no apparent defect (8).

Conversion of cholesterol into 7DHC is catalyzed by the evolutionary conserved Rieske-domain oxygenase Neverland (NVD) in insects and nematodes (9, 10). To test whether mutation(s) in *nvd* are responsible for *D. pachea* dependence upon its host cactus, we sequenced the *nvd* coding region (8) from *D. pachea* and the three most closely related species *D. nannoptera*, *D. acanthoptera* and *D. wassermani*, which feed on other cacti (11) (Table S1–2, fig. S1). No stop codon or insertions/deletions were found in the *D. pachea* sequence, but the ratio of rates of nonsynonymous substitution (d_N) over synonymous substitution (d_S) is significantly higher in the branch leading to *D. pachea* (Tables S3, fig. S2). We noticed that several amino acids showing high conservation across insects and vertebrates are different in *D. pachea* NVD (Fig. 1B–C). We observed that in *D. pachea* third instar larvae, as in *D. melanogaster* (9) and *D. acanthoptera*, *nvd* is only expressed in the prothoracic gland (fig. S3), an organ whose sole known function is ecdysone production (12). Therefore we conclude that NVD function, if any, should be related to steroid hormone production.

The senita cactus does not contain cholesterol nor 7DHC but does produce three other sterols – lathosterol, campestenol and schottenol (6) – which, if used as precursors for steroid hormone synthesis, are expected to lead to different steroid hormones, respectively 20-hydroxyecdysone, makisterone A and makisterone C (fig. S4), due to the inability of *Drosophila* to dealkylate phytosterols (13). Steroids from *D. pachea* extracts were separated by HPLC and fractions of interest were analyzed with mass spectroscopy. We detected ecdysone and 20-hydroxyecdysone but no trace of makisterone A or makisterone C (fig. S5). These results suggest that *D. pachea* only uses lathosterol and not the other senita cactus sterols as steroid hormone precursors.

Since conversion of cholesterol into 7DHC biochemically resembles the transformation of lathosterol into 7DHC (Fig. 1A), we hypothesized that *D. pachea* NVD converts lathosterol rather than cholesterol into 7DHC (14). To test this hypothesis, we generated transgenic *D.*

melanogaster flies in which the endogenous *nvd* gene is shut down by RNA interference (RNAi) and replaced by *D. pachea nvd*. The *D. melanogaster nvd* RNAi flies do not develop on regular fly food (9) nor on food supplemented with lathosterol, yet they reach the adult stage on regular fly food supplemented with 7DHC (9) (Fig. 2, Table S4). As expected, introduction of *D. pachea nvd* into *D. melanogaster nvd* RNAi flies rescues development on food supplemented with lathosterol but not with cholesterol (Fig. 2). This demonstrates that *D. pachea* NVD can use lathosterol but not cholesterol as a substrate (Fig. 1A).

To identify the amino acid changes responsible for the loss of *D. pachea* NVD activity with cholesterol, we reconstructed ancestral NVD sequences (8) for the entire protein region except for the N-terminal region, which does not show conserved amino acid sequence among insects. Interestingly, we found 19 mutations in the lineage leading to *D. pachea*, of which five are predicted to affect protein function (Fig. 1C). We sequenced the entire *nvd* coding region in three *D. pachea* strains and in two natural population samples. The five predicted functionally relevant amino acids were found in all the 32 individuals. To test whether these five amino acid changes affect NVD activity, we established an *in vitro* assay of NVD activity with GFP-control and NVD constructs (8, 15). GFP-transfected cells produced no 7DHC whereas cells transfected with *nvd* from various insects including *D. acanthoptera* converted cholesterol and lathosterol into 7DHC (Fig. 3A, S6). In accordance with our *D. melanogaster* transgenic assays, we observed that cells transfected with *D. pachea nvd* do not convert cholesterol into 7DHC but convert lathosterol into 7DHC (Fig. 3A, S6), although at a lower level relative to other species. Control experiments with HA epitope-tagged NVD constructs revealed that *D. pachea* NVD accumulates at similar levels as the other NVD homologs in the in vitro assay (fig. S7). These results indicate that the ancestral *Drosophila* NVD enzyme was likely able to transform both cholesterol and lathosterol into 7DHC and that NVD has subsequently lost the ability to convert cholesterol in the lineage leading to *D. pachea*.

We tested the effect of the five predicted functionally relevant amino acid changes by introducing each mutation individually in the *nvd* sequence from *D. mojavensis*, another cactophilic species endemic to the Sonoran desert, which displayed the highest in vitro NVD activity. With either cholesterol or lathosterol as a substrate, substitution P290C slightly increased the activity, G376T and L300I decreased the activity by half, and substitutions G250A and E377G reduced the activity to less than 18% of the wild-type activity (Fig. 3B, S7). We also performed the reciprocal experiment and reintroduced the predicted ancestral amino acid residues into the *D. pachea* NVD sequence. We found that a NVD activity close to that of *D. acanthoptera* is not restored by a single amino acid change but by four amino acid changes in concert (Fig. 3C, S7). Corroborating these in vitro results, introduction of a *D. pachea nvd* construct containing these four amino acid changes into *D. melanogaster nvd* RNAi flies rescues development on food supplemented with cholesterol (Fig. 2). We conclude that two to four mutations in the *D. pachea nvd* coding region have caused the loss of NVD activity with cholesterol substrate. These mutations have turned *D. pachea* into an obligate specialist dependent on lathosterol, a compound that has been found in a single plant species in the Sonoran desert (5, 6).

Remarkably, *D. melanogaster nvd* RNAi flies expressing *D. pachea nvd* survive significantly better on lathosterol than on cholesterol (t-test, $t_{10,11} = 2.029$, $p < 0.03$, Fig. 2) but no effect on survival was detected with *nvd* RNAi flies expressing *D. pachea nvd* with the four ancestral amino acid changes (Fig. 2). This suggests that the mutations that abolished cholesterol conversion during *D. pachea* evolution provide a fitness advantage on lathosterol. The underlying mechanism remains unclear. Our in vitro assay does not uncover any benefit from the *D. pachea nvd* mutations: *D. pachea* NVD in vitro activity with lathosterol is not higher compared to other species (Fig. 3) and the NVD enzymes of related *Drosophila* species are already able to convert lathosterol into 7DHC. To assess population genetic forces at play on the *nvd* genomic region, we compared the 3-kb *nvd* locus and 7 genes on the same 100-kb-scaffold with 9 control genes in 34 individuals from a single natural population. Our analysis reveals that *nvd* is in a genomic region of low nucleotide diversity, low recombination rate and normal divergence rate (MK test, $p > 0.85$; MLHKA test, $p < 10^{-5}$, fig. 4, Tables S5–11). A signature of selective sweep (Kim and Stephan W) is detected over *nvd* and neighboring loci (fig. 4), but nucleotide polymorphism is too low to infer whether this recent selection acted on the *nvd* mutations themselves. Tajima's D and Fu and Li tests are consistent with recovery from selective sweep in the *nvd* region (Table S6).

A likely scenario is that *D. pachea* first evolved a resistance towards senita cactus toxic compounds and slowly became restricted to this food source as it escaped competition with other fly species. Evolution of *D. pachea's* resistance most likely did not involve NVD since *nvd* is not expressed in the midgut and fat body (fig. S3), the detoxification organs in insects (16). As lathosterol became *D. pachea*'s unique source of sterols for steroid hormone synthesis, mutations in *nvd* that abolished NVD activity on cholesterol appeared and fixed rapidly due to their beneficial effect with lathosterol. As a result, *D. pachea* became an obligate specialist on the senita cactus. We point out that besides *nvd* mutations, mutation(s) in other genes might also have contributed to *D. pachea* dependence on lathosterol. Alternatively, the identified *nvd* mutations may have spread while *D. pachea* ancestors were still feeding on various plants and may thus have accelerated its ecological specialization. Our study, which uncovered several mutations underlying the obligate bond between a specialist species and its host, illustrates how a few mutations in a single gene can restrict the ecological niche of a species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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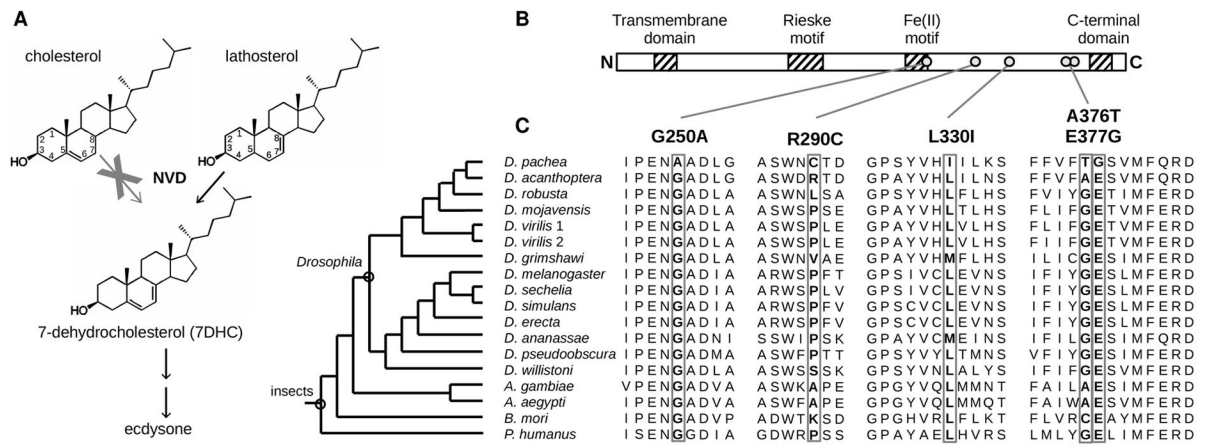
Fig. 1.
Presumed ecdysone biosynthetic pathway (**A**) and NVD in *D. pachea*. (**B**) NVD protein structure. (**C**) Alignment of multiple NVD protein sequences. Five mutations (boxes) were tested in vitro. For an alignment of full NVD protein sequences with additional insect species see fig. S8.
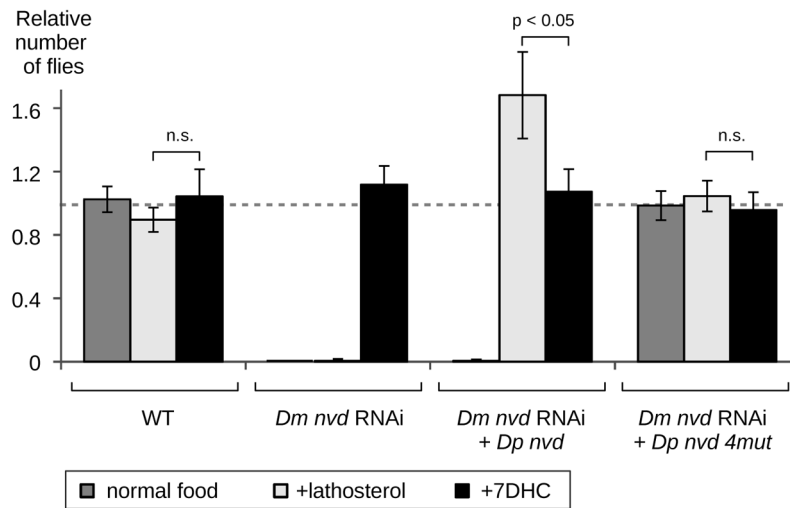
Fig. 2.
Fly survival on various food media. *WT*: control flies, *Dm nvd* RNAi: RNAi knockout of wildtype *D. melanogaster nvd, +Dp nvd*: rescued with wildtype *D. pachea nvd, +Dp nvd 4mut*: rescued with *D. pachea nvd A250G I330L T376A G377E*. The proportion of flies of each genotype is indicated relative to the number of *UAS-nvd RNAi Sb* male siblings (*8*, Table S4). Bars show average and error bars are SE.
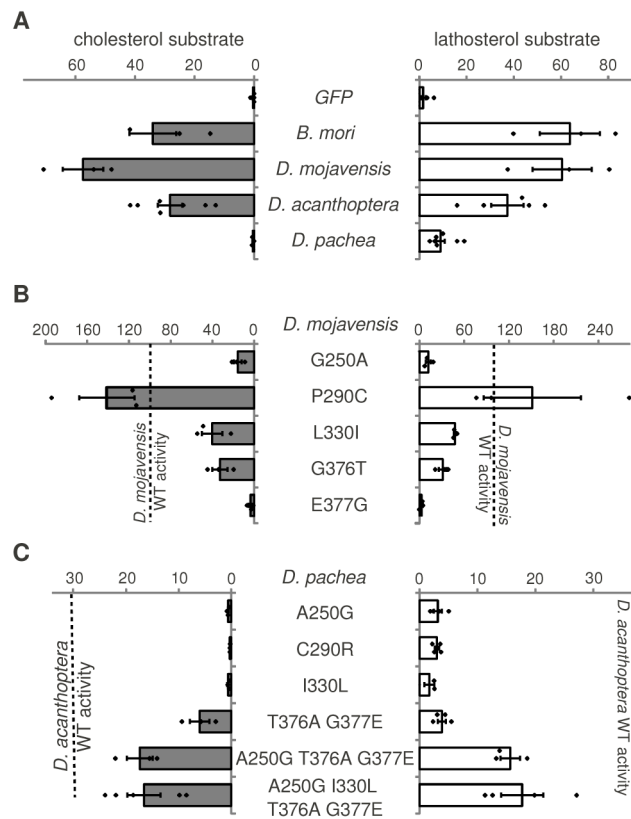
Fig. 3.
NVD enzyme activity with cholesterol (left, grey) or with lathosterol (right, white). (**A**)
wildtype (WT) NVD enzymes. (**B**) *D. mojavensis* NVD enzymes containing single
mutations. (**C**) *D. pachea* enzymes containing reverse mutations. Bars represent average
activity, error bars SD, and dots data points. Note that two *D. mojavensis nvd* wild-type
constructs were used in our assays. Enzyme activity is indicated as a percentage relative to
the NVD activity obtained with a *D. mojavensis nvd* construct that includes the *nvd* gene
5′UTR (9). All the *D. mojavensis* constructs tested in (**B**) contained this 5′UTR. The dotted
line indicates *D. mojavensis* NVD wild-type activity (construct containing the 5′UTR) in (**B**)
and *D. acanthoptera* NVD WT activity in (**C**).
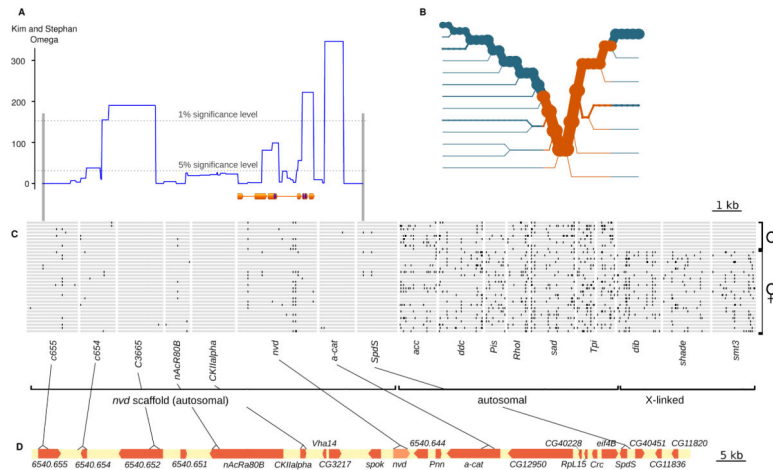
Fig. 4.

The *nvd* region is under positive selection. (**A**) Kim and Stephan's omega statistics across the *nvd* region. Omega values above the significance level indicate a selective sweep. The *nvd* coding regions are represented below, with the position of the five tested amino acid changes in purple. (**B**) Haplotype bifurcation plot. Circles indicate polymorphic sites in the *nvd* gene (orange) and in neighboring loci (blue). Line thickness is proportional to the number of samples with the indicated haplotype. (**C**) Representation of the genotypes of 34 individuals. Black bars indicate heterozygote positions. Homozygote sites for rare alleles are not shown. (**D**) Position of the sequenced loci within the *nvd* region. Gene annotations are in orange.