Pacific Science (1997), vol. 51, no. 4: 475–489

Adaptive Radiation in the Hawaiian *Drosophila* (Diptera: Drosophilidae): Ecological and Reproductive Character Analyses[1]

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ABSTRACT: The entomologist R. C. L. Perkins pioneered observations of breeding site ecology for the endemic Hawaiian Drosophilidae, a renowned group of flies that has undergone explosive speciation and adaptive radiation into a wide variety of breeding niches. Females of the various species groups and subgroups oviposit their eggs in either fungi, flowers, fruits, leaves, stems, bark, sap fluxes, or other novel substrates. Varied selective forces in these alternative breeding sites have apparently molded female reproductive characters and strategies into diverse outcomes; some species mature and oviposit only one egg at a time, whereas others oviposit hundreds. Here, we have analyzed the pattern of shifts in breeding substrate, and the associated evolution of selected ovarian, egg, and ovipositor traits, by mapping the various ecological and female reproductive character states on an independently derived phylogenetic hypothesis based on nuclear and mitochondrial DNA sequences. This comparative phylogenetic approach demonstrates a number of strong historical associations among female reproductive traits and between particular traits and the breeding substrate, although the overall pattern is complex and more data are needed. Identification of certain apomorphic traits associated with shifts in breeding substrate suggests an adaptational origin for some of the changes in egg load per fly, in the length of the respiratory filaments of the egg, and in the length and shape of the ovipositor. Although these hypotheses need further testing, it appears that the ecological diversification in breeding substrates has been an integral component in the radiation of drosophilids in Hawai'i.

OF ALL THE FASCINATING groups of insects collected by R. C. L. Perkins in his historic expeditions of the 1890s to survey the fauna of the Hawaiian Islands, none is more speciose and more diverse than the drosophilids. By his own admission, Perkins paid relatively little attention to the "minute and obscure Diptera," yet he recognized that the most striking feature of the dipterous fauna was "the extraordinary development of the Drosophilidae, in structure and in the number of species," which he moderately estimated as about 300 (Perkins 1913), although his collections were limited to some 47 new species. In a preliminary taxonomic study of the Hawaiian Drosophilidae, Hardy (1965) described 400 species from Hawai'i, noting that the Hawaiian drosophilid fauna was without doubt one of the most remarkable of any area of the world and "by far the largest known group of animals in Hawai'i." With current estimates of more than 800 endemic species (Hardy and Kaneshiro 1981; Kaneshiro, pers. comm.), the Drosophilidae must certainly be considered a dominant component of the Hawaiian insect fauna. What is most remarkable is that this morphologically and behaviorally diverse group of flies probably evolved from one original founder or at most two founder individuals from a distant continent (Throckmorton 1966). Indeed, Perkins (1913) understood that many well-developed groups in Hawai'i had evolved from a single successful immigrant and was ahead of his time in enunciating some of the basic aspects of founder effect speciation (Mayr 1954, Carson

[1] Presented at Symposium 2S-4: The Legacy of R. C. L. Perkins: 100 Years of Hawaiian Entomology (XX International Congress of Entomology, 27 August 1996). This research was supported by NSF grant BSR 89-18650 to the authors. Manuscript accepted 3 February 1997.

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1971), including the extreme rarity of successful immigration and the role of isolation in triggering the founder population to produce a new species.

Perkins was a remarkably observant and insightful biologist. Not only did he recognize the central role of geographic isolation in the evolution of Hawaiian organisms, but he also noted as a striking feature of the fauna "the tendency of island creatures to limit their range and to specialize their habits" (Perkins 1913), foreshadowing the concept of adaptive radiation and the intimate association between each organism and its microenvironment. In questioning why some groups have proliferated more than others, and, in particular, attempting to identify what has been responsible for the extraordinary development of the Hawaiian *Drosophila,* the biological basis of the adaptive radiation of the group is a key feature to investigate. Because reproductive success is critical to evolutionary success, our particular focus is on the diversification of the female reproductive system in the context of the ecological diversification in oviposition sites of the endemic Hawaiian *Drosophila.* In our view, this is one of the key elements in their evolution. This focus on the variation in female reproductive characters of Hawaiian drosophilids fits in with the broader aim of our research program, which is to integrate ecological, developmental, morphological, and molecular analyses with modern phylogenetic approaches, to arrive at a better understanding of the evolution and adaptive radiation of this most spectacular group of Hawaiian insects.

Although Perkins had no inkling of the enormous diversity in female reproductive strategies among the Hawaiian Drosophilidae (Kambysellis and Heed 1971), he did note their diversity in breeding habits. His comments in his review of the Drosophilidae in the *Fauna Hawaiiensis* reveal his keen powers of observation: "Some of the species are quite conspicuous, and are readily attracted by the sap oozing from a broken limb of a tree, or from exudations caused by decay or disease. Very many breed in stems of trees or plants, which, when decaying, yield abundant moisture—such as those of the arborescent lobelias, of bananas, tree-ferns, etc. The larvae abound also beneath the bark of some forest-trees, which, when this is stripped off, reveal a semiliquid or pulp-like material covering the wood. Some of the larger and very many of the smallest and most obscure species live amongst the soft ferns, which grow in damp places beneath the shade of the forest-trees" (Perkins 1913:189). Here Perkins correctly described the habitats of Hawaiian *Drosophila,* identifying tree fluxes, decaying stems, and bark as prime oviposition sites. It is interesting that he was even aware of the role of the arborescent lobeliads as an important resource for these flies. More detailed ecological studies (Heed 1968, 1971, Montgomery 1975) have confirmed Perkins' initial observations and expanded knowledge of Hawaiian drosophilid breeding sites to also include fungi, decaying flowers, leaves, fruits, and roots, with some 40 families of endemic Hawaiian plants being used for oviposition. As well as utilizing a diverse array of subcortical, endophytic, and exophytic oviposition sites, a few Hawaiian drosophilids are even reported as endozoic: specifically, those that are parasitic on spiders' eggs (Hardy 1965, Heed 1968).

Successful exploitation of this heterogeneous array of breeding substrates has apparently required modification of several aspects of the female reproductive system. Because of varying nutritional resources in these oviposition sites that limit the number of larvae that can be supported, oviposition behavior, and ovarian structure and function have evolved to better match egg output with the carrying capacity of the breeding site (Kambysellis and Heed 1971). The ultrastructure of the eggs themselves (Kambysellis 1993) and the morphology of the ovipositor (Throckmorton 1966; L. Franchi, P. Francisco, M.P.K., and E.M.C., unpubl. data) are also extremely variable, and the observed specializations of the ovipositors and eggs of individual species might be interpreted as the outcome of natural selection for successful oviposition and embryonic development in the particular breeding site used by each species.

To analyze the female reproductive variation evident among Hawaiian *Drosophila* and better understand the evolution and adaptation of individual traits, we have taken a cladistic approach. We use an independent phylogenetic hypothesis to first trace the sequence of ecological shifts in breeding substrates, and then the sequence of

evolutionary shifts in individual reproductive characters, to explore the patterns of evolution in each trait. The historical associations among traits and between the morphological traits and breeding substrates are then evaluated by comparison of the patterns of phylogenetic character change. This phylogenetic method of analyzing character evolution provides a powerful approach to documenting adaptation (Coddington 1988) and demonstrates that divergence in ovipositional behavior and in the female reproductive system has played an important role in the radiation of the Hawaiian Drosophilidae.

MATERIALS AND METHODS

Phylogenetic analysis of the evolution of selected ecological and female reproductive characters was performed by mapping the alternate character states (considered as unordered) on an independently derived phylogenetic hypothesis using the program MacClade (Maddison and Maddison 1992). In each case, we examined the parsimony reconstruction, and as well, the delayed transformation (DELTRAN) optimization option, which maximizes parallel changes, and the accelerated (ACCTRAN) tracing, which maximizes reversals, in an attempt to resolve some of the ancestral nodes. The tree used for these analyses was a composite molecular cladogram of 44 Hawaiian drosophilid species described more fully in Kambysellis and Craddock (1997) and based on parsimony analysis (Swofford 1993) of sequences of the *Yp1* Yolk protein gene (Kambysellis et al. 1995) and a set of nuclear and mtDNA sequences (Baker and DeSalle 1997).

The characters analyzed here included one ecological and five female reproductive traits. Data on the type of breeding substrate selected by females for oviposition were taken from the rearing records of Heed (1968) and Montgomery (1975). The substrates included fungi, decaying leaves, stems, bark, and sap fluxes. In cases where a drosophilid species had been recorded from more than one substrate class, the predominant substrate was chosen for this analysis. The predominant substrate used in nature was identified as that particular substrate that produced more than 50% of the emerging adult individuals; usually the percentage was much higher. For *D. grimshawi* Oldenberg from Maui, there was no one predominant substrate among the multiple substrates recorded, and hence this species was coded as a multi-substrate user.

Female reproductive characters included selected ovarian, egg, and ovipositor traits. As an index of female fecundity, we estimated the egg load in the ovaries (more explicitly, the number of mature eggs per fly), using data on the number of ovarioles per fly and the number of mature eggs per ovariole taken from field-collected individuals (Kambysellis and Heed 1971; M.P.K. and E.M.C., unpubl. data). The data were classified into six character states as follows: 0 = 1–5 eggs per fly; 1 = 6–15 eggs per fly; 2 = 16–25 eggs per fly; 3 = 26–50 eggs per fly; 4 = 51–100 eggs per fly; and 5 = >100 eggs per fly. The egg traits analyzed were (1) the length of the egg (0 = 0.6–0.9 mm; 1 = 0.91–1.20 mm; 2 = 1.21–1.50 mm; 3 = >1.50 mm); and (2) the relative length of the respiratory filaments or dorsal appendages (Spradling 1993) that project from the anterior end of the egg. These latter data refer to the length of the longer, more posterior pair of filaments relative to the length of the egg. Relative filament length was coded as follows: 0 = filaments absent; 1 = very short filaments, <0.1 times the length of the egg; 2 = moderate-length filaments, 1–1.4 times the length of the egg; and 3 = long filaments, 1.5 times or >1.5 times the length of the egg. Data on egg traits were taken from Kambysellis and Heed (1971) and Kambysellis (1993, and unpubl. data).

Scanning electron microscopy (SEM) was used to make observations on the ovipositor, the female ovipositional organ at the posterior end of the abdomen ventral to the anal plate. Mature female flies were first chilled in the freezer, which generally induces extrusion of the ovipositor from the abdomen. Alternatively, the abdomen was squeezed to cause full extension, and then the posterior end of the abdomen was dissected. For dry museum specimens, the abdomen was removed and rehydrated in insect Ringer's before dissecting the ovipositor. Following clearing in phenol and pine oil, the specimen was mounted on a stub, sputter-coated with gold/palladium, and observed at accelerating voltages of up to 30 kV, using a Model ISI-SR-50 SEM.

Micrographs of lateral and ventral views of the ovipositor of each species were prepared, as well as a higher magnification of the tip of the ovipositor. Measurements were made of the length and width of the ovipositor when viewed laterally, for many species directly using the SEM, or alternatively from the micrographs. In addition, thorax length was measured, as an index of body size, because ovipositor length may not be independent of the body size of the fly.

For the phylogenetic analysis of raw ovipositor length, four character states were distinguished: 0 = very short (<250 μm in length); 1 = short (300–425 μm); 2 = medium (425–550 μm); and 3 = long (>550 μm). The ratio of the length of the ovipositor to the maximum width of the ovipositor (L/W) was calculated as a crude index of ovipositor shape, and this value was used, together with the shape of the ovipositor tip, and the overall size, to group ovipositors into seven "shape classes." These were coded as follows: 0 = very short and ovoid (L/W = 1–2; L <235 μm, tip rounded); 1 = oblong-shaped (L/W = 2–3.6; L = 300–450 μm; tip rounded); 2 = canoe-shaped (L/W = 4.5–6; L = 450–720 μm; tip very sharp and pointed with a distinctive spike on the end); 3 = ovoid (L/W ~ 3.5; L ~ 500 μm; pointed tip); 4 = triangular (L/W = 2.0–2.7; L = 400–600 μm; tip tapered); 5 = oblong (L/W = 3.2–4.5; L = 450–500 μm; tip blunt); and 6 = long and slender ovipositor (L/W >5; L = 500–950 μm; tip narrowed but not sharp).

RESULTS AND DISCUSSION

Hawaiian Drosophilid Breeding Substrates and Pattern of Ecological Shifts

The array of substrates chosen by Hawaiian drosophilid females for oviposition and larval development encompasses fungi and decaying parts (leaves, stems, bark, flowers, fruits, roots) of specific Hawaiian plants, as well as sap fluxes of certain trees, and, in the case of a few species, animal material in the form of spiders' eggs (Hardy 1965, Heed 1968, Montgomery 1975). Females are generally highly consistent in their choice of oviposition site; very few species use multiple substrates (Heed 1968, 1971, Montgomery 1975, Kambysellis and Craddock 1997). The ecological diversity among Hawaiian drosophilids is not random but follows a defined pattern that can be elucidated by a cladistic analysis of breeding substrate use. This requires a working phylogenetic hypothesis. The relationships among species and lineages of Hawaiian drosophilids are best defined by the recently available molecular phylogenies (Thomas and Hunt 1991, Kambysellis et al. 1995, Baker and DeSalle 1997, Kambysellis and Craddock 1997). Although these phylogenies based on nuclear and mtDNA sequences include a limited number of species with respect to the large size of the group, nonetheless, because they include representative species of the major species groups and subgroups, they portray the main interrelationships and provide an overall assessment of Hawaiian drosophilid phylogeny. Although not completely concordant in every detail, the molecular phylogenies are in broad general agreement with inferences as to evolutionary relationships of the Hawaiian drosophilids based on morphological (Throckmorton 1966), behavioral (Spieth 1982), and polytene chromosomal analyses (Carson and Yoon 1982, Carson 1992). Even where past hybridization is suspected, as between the two closely related species *D. silvestris* and *D. heteroneura,* the effects on molecular characters are expected to be similar to those on other characters, so the molecular phylogeny should be no more biased than other phylogenies.

To elucidate the series of ecological shifts in breeding substrates, we traced predominant substrate use on a composite molecular tree of 44 Hawaiian species rooted with two non-Hawaiian species, *D. mulleri* Sturtevant and *D. melanogaster* Meigen, as outgroups (Kambysellis and Craddock 1997). The results of this ecological character mapping are displayed in Figure 1, which shows that clades are generally ecologically homogeneous, although there are several instances of ecological shifts within groups and subgroups, especially within the more derived clades. Thus, among the most primitive drosophiloid groups, the white-tip scutellum flies are fungus breeders, whereas the modified-tarsi and the antopocerus groups are leaf breeders; clearly, the shift to utilizing decaying leaves initiated a phylogenetically distinct lineage. (The even more primitive scaptomyzoid group is poorly

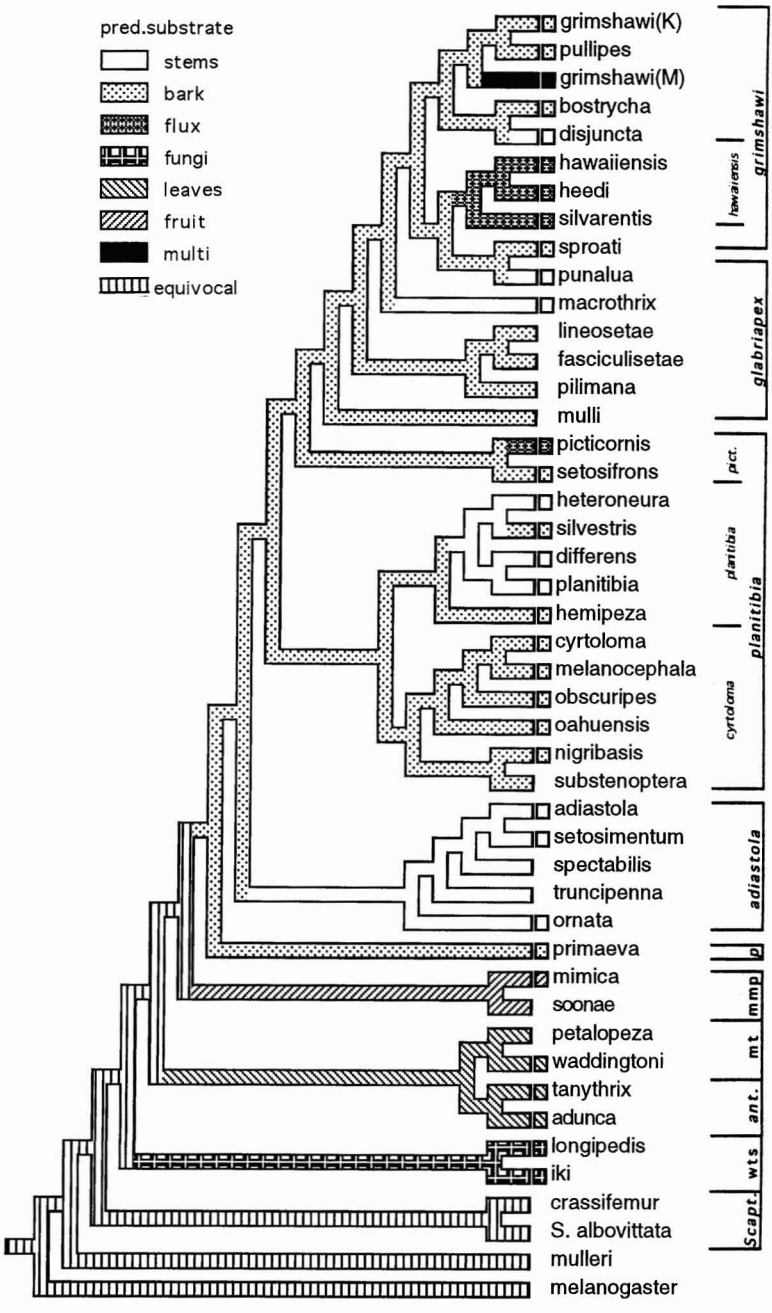

FIGURE 1. Pattern of ecological diversification in breeding substrates among the Hawaiian drosophilids. The character analyzed here is the predominant substrate used by each species for oviposition and larval development (see key and Materials and Methods). Phylogenetic character mapping on the molecular tree presented in Kambysellis and Craddock (1997) was performed using the MacClade program (Maddison and Maddison 1992) with the accelerated transformation optimization implemented. The ecological data were taken from Heed (1968) and Montgomery (1975). Species for which no ecological data are available lack the small box at the end of the branch. Species are clustered into the formerly recognized species groups (in bold) and subgroups bracketed to the right of the tree. Reading from the bottom up abbreviations are as follows: *Scapt.* (the scaptomyzoid group); wts (white-tip scutellum group); *ant.* (antopocerus group); mt (modified-tarsi group); mmp (modified-mouthparts group); *p* (*primaeva* species group—this and all the above species are included in the picture-wings); *pict.* (the *picticornis* subgroup of the *planitibia* species group). The K and M following *D. grimshawi* refer, respectively, to the Kaua'i and Maui populations, which recently have been recognized as distinct species. The Kaua'i population will be named as a new species (Kaneshiro, pers. comm.).

characterized taxonomically and ecologically, so is not analyzed here.) *Drosophila mimica* Hardy of the modified-mouthparts group is predominantly a fruit breeder, but this group is ecologically diverse, with other species breeding in leaves, stems, flowers, or fungi (Heed 1968). More studies are required on the phylogenetic relationships and ecology of this large group, but it is clear that the evolution of the modified-mouthparts group began a radiation into novel ecological substrates such as decaying stems and fruits that had not previously been used by Hawaiian drosophiloids.

Further ecological shifts followed the evolution of the more derived picture-winged group, a better-analyzed assemblage of some 110 species, of which we include 34 species on the molecular tree. Two new substrates invaded by this group are decaying bark and tree fluxes. Some of the picture-winged species groups and subgroups, however, have retained the use of decaying stems (Montgomery 1975), an adaptation that first appeared in the modified-mouthparts group (Heed 1968). There appear to have been two independent shifts to flux breeding in the picture-wings, one in the *planitibia* species group involving the species *D. picticornis* Grimshaw, and the other in the *grimshawi* species group, entailing the whole *hawaiiensis* species subgroup. This shift from bark breeding to flux breeding might be considered a more minor ecological shift than the previous shifts, because in most cases the subcortical breeding site is maintained. In the former case, larvae develop in decaying bark; in the latter, larvae develop under bark of a living tree that has been moistened by the flux drippings. Even so, not all flux habitats are homologous. The larvae of *D. picticornis,* which occupy fluxes under the bark of *Metrosideros* trees, are closely associated with a native leafhopper that produces copious amounts of honeydew (Montgomery 1975). Further, the "soil flux" occupied by *D. heedi* Hardy & Kaneshiro (see below) is very different from the tree fluxes used by other members of the *hawaiiensis* subgroup. Initial designation of all these *Drosophila* species as flux breeders obscures some important distinctions; probably, a finer ecological classification is required to fully understand the biological and evolutionary features of these flies.

Although *D. primaeva* Hardy & Kaneshiro has been recorded as a bark breeder (Montgomery 1975), suggesting that the shift to using decaying bark coincided with the origin of the picture-winged assemblage (Figure 1), several of the female reproductive traits of this species are not at all characteristic of bark-breeding species (see below). Reinvestigation of the ecology of this species is thus in order. If it is confirmed that *D. primaeva* is not a bark breeder, but rather a stem breeder, as we suspect, then the shift to bark breeding must have occurred later in the evolution of the picture-wings, associated with the evolution of the *planitibia* species group. The stem-breeding habit of the *adiastola* species group would not then appear as a reversal; rather, stem breeding would be viewed as a conserved trait, retained from its first appearance in the modified-mouthparts group (Heed 1968), through the evolution of the *primaeva* and *adiastola* species groups. Nonetheless, a number of reversals from bark breeding to stem breeding must still be inferred in the later evolution of the picture-wings (Figure 1), the exact number and pattern depending on choice of the accelerated or delayed transformation optimization.

Evolution of Ovarian and Egg Traits

The Hawaiian Drosophilidae display a wide range of reproductive capabilities, with females of some species maturing and ovipositing only one egg at a time, whereas other species can oviposit several hundred eggs at a time (Kambysellis and Heed 1971). Although oviposition behavior varies, with eggs deposited in or on the substrate either singly or in clusters, female fecundity is largely a function of two ovarian parameters—the number of functional ovarioles per ovary and the maximum number of mature eggs per ovariole. The more variable of these traits pertains to ovarian structure, the number of ovarioles per fly (see Table 1) being a major controlling factor of female fecundity. The majority of species have only one mature egg per ovariole, but some species can mature two or three eggs per ovariole (Kambysellis and Heed 1971). As a measure of female fecundity, we have mapped potential egg load (the number of ovarioles per fly times the number of mature eggs per ovariole) on the phylogenetic hypothe-

TABLE 1

VARIATION IN SIZE, OVARIAN, EGG, AND OVIPOSITOR TRAITS AS A FUNCTION OF ECOLOGICAL SUBSTRATE AMONG A SAMPLE OF HAWAIIAN DROSOPHILIDS (TWO FROM THE GENUS *Scaptomyza* AND 17 *Drosophila* SPECIES)

SPECIES	BREEDING SUBSTRATE	MEAN THORAX LENGTH[a] (in mm)	MEAN NO. OF OVARIOLES PER FLY[a]	MEAN EGG LENGTH[a] (in mm)	LENGTH OF POSTERIOR FILAMENTS[a] (in mm)	OVIPOSITOR LENGTH (in mm)	OVIPOSITOR SHAPE (L/W)
*S. albovittata**	?	0.92 ± 0.04	—	0.61 ± 0.02	0	0.083	1.1
S. caliginosa	Flowers	0.93 ± 0.08	2.50 ± 0.29	0.88 ± 0.03	0	0.207	2.8
D. waddingtoni	Leaves	1.45 ± 0.10	11.79 ± 1.53	0.90 ± 0.03	0.83 ± 0.02	0.459	5.7
D. adunca	Leaves	2.94 ± 0.12	11.00 ± 1.23	1.61 ± 0.04	1.56 ± 0.14	0.715	4.9
D. diamphidiopoda	Leaves	2.28 ± 0.13	18.44 ± 1.06	1.09 ± 0.04	1.23 ± 0.09	0.502	6.6
D. kambysellisi	Leaf surface	1.51 ± 0.08	15.00 ± 1.23	0.79 ± 0.02	0.63 ± 0.04	0.344	3.9
D. mimica	Fruit	1.78 ± 0.18	23.85 ± 4.21	0.74 ± 0.02	0.61 ± 0.04	0.331	2.9
D. primaeva	Bark?	3.00 ± 0.12	101.33 ± 8.44	0.83 ± 0.02	0.67 ± 0.08	0.528	3.4
D. truncipenna	Stems	3.22 ± 0.13	48.00 ± 4.90	0.96 ± 0.04	0.68 ± 0.06	0.533	2.1
D. clavisetae	Stems	2.71 ± 0.11	38.17 ± 3.56	0.99 ± 0.03	1.19 ± 0.07	0.550	2.0
D. adiastola	Stems	2.41 ± 0.09	45.92 ± 7.25	0.82 ± 0.02	0.97 ± 0.04	0.457	2.7
D. picticornis	Sap flux	1.77 ± 0.10	27.44 ± 3.65	0.81 ± 0.03	1.44 ± 0.02	0.510	5.1
D. melanocephala	Bark	3.31 ± 0.34	86.60 ± 9.24	0.90 ± 0.03	1.35 ± 0.12	0.750	6.4
D. silvestris	Bark	3.16 ± 0.13	52.38 ± 2.67	0.94 ± 0.03	1.78 ± 0.15	0.767	8.7
D. pilimana	?	2.19 ± 0.17	45.00 ± 3.74	0.75 ± 0.01	0.59 ± 0.03	0.482	3.2
D. fasciculisetae	?	2.65 ± 0.22	47.22 ± 6.36	0.77 ± 0.02	0.73 ± 0.05	0.655	7.8
*D. silvarentis**	Bark flux	3.08 ± 0.28	39.29 ± 0.63	0.86 ± 0.02	1.70 ± 0.05	0.584	6.8
D. sproati	Bark	2.78 ± 0.15	65.55 ± 5.93	0.87 ± 0.02	2.36 ± 0.13	0.720	6.4
*D. mulli**	?	2.20	41.25 ± 0.83	1.00 ± 0.02	0.31 ± 0.02	0.727	7.9

[a] Species means and standard errors for thorax size, egg, and ovarian traits taken from Kambysellis and Heed (1971), except in the case of the three species marked with an asterisk.

sis. The resulting evolutionary pattern is shown in Figure 2*a*. The most primitive scaptomyzoid lineage produces only a few eggs at a time. The next more derived groups, the fungus-breeding white-tip scutellum flies, leaf breeders of both the antopocerus and modified-tarsi lineages, and the modified-mouthparts group have a moderate number of ovarioles (Table 1) and intermediate fecundity (up to 25 eggs per fly), but generalizations are perhaps premature in view of the limited sampling of these primitive groups. All the picture-winged flies have higher fecundities (>25 eggs per fly), as a result of the ability of some species to mature more than one egg per ovariole and their higher ovariole numbers, which range up to 101 ovarioles per fly in *D. primaeva* (Table 1 [Kambysellis and Heed 1971]). Although reproductive data are incomplete, fecundity levels tend to be clade-specific, but with some notable exceptions (Figure 2*a*).

Kambysellis and Heed (1971) proposed that the predictability and the carrying capacity of the larval habitat are major factors influencing fecundity potentials. Thus it appears that the primitive flower-breeding scaptomyzoid species, which occupy a rather ephemeral habitat, have the lowest potential; the leaf-breeding species, which utilize a somewhat more nutritious and dependable resource, have an intermediate potential; and the stem-breeding and bark-breeding species, which use nutritionally richer and longer-lasting resources, have the highest potential, with bark breeders generally having even higher egg loads than stem breeders. (Fungus breeders and flux breeders were not considered in those earlier studies.) The phylogenetic analysis reveals that overall there has been a progressive increase in egg load per fly, as new niches of apparently greater carrying capacity were invaded in the evolutionary diversification of this group of flies in Hawai'i.

Variation in egg size is relatively minor among Hawaiian *Drosophila,* and the phylogenetic analysis failed to reveal any distinctive pattern. However, variation in the length of the respiratory filaments at the anterior end of the egg is truly striking. At one extreme are eggs with no respiratory filaments at all, as in *D.*

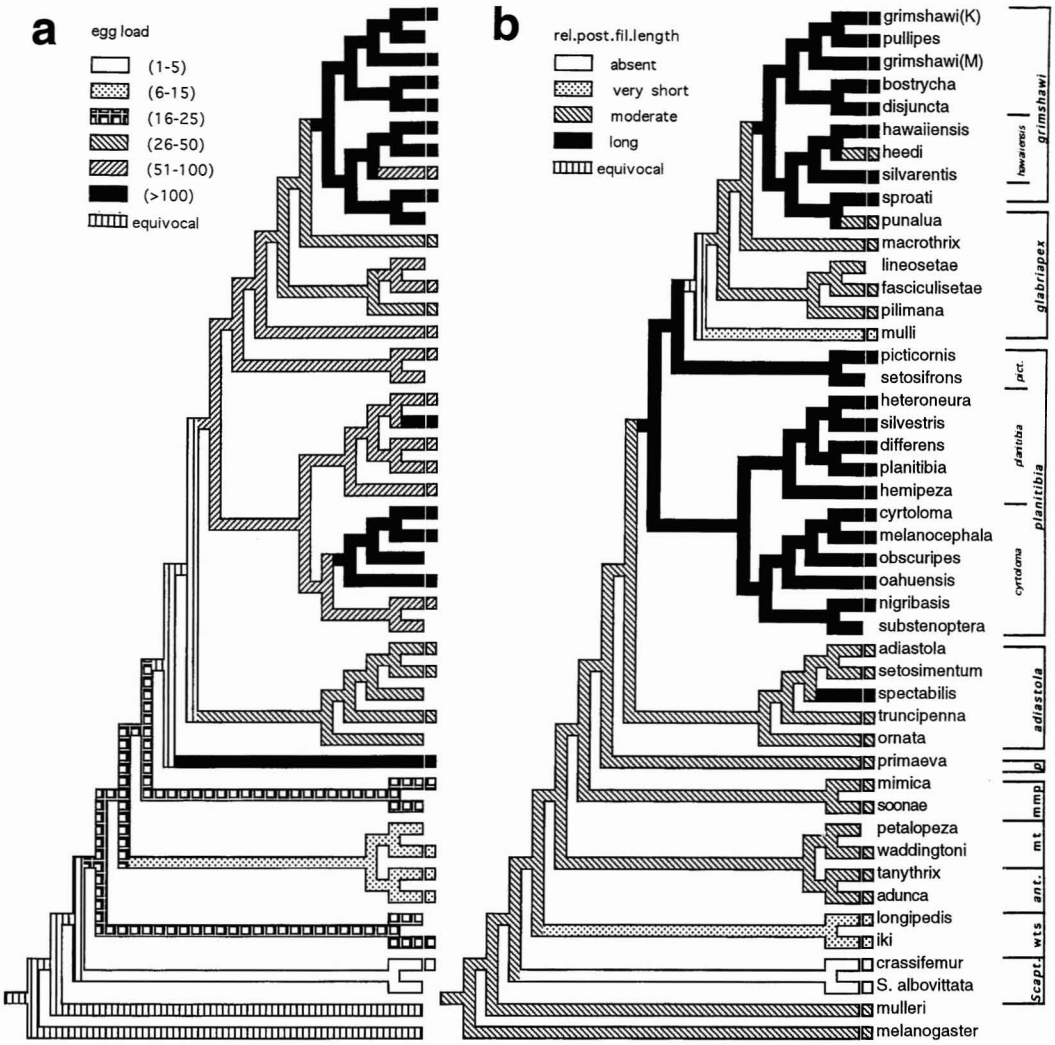

FIGURE 2. Phylogenetic pattern of evolution of two female reproductive traits among Hawaiian drosophilids. *a.* Egg load per fly; *b.* Length of the posterior respiratory filaments relative to the length of the egg (see keys and Materials and Methods for further details of the character classes). Data are from Kambysellis and Heed (1971) and unpubl. For both traits, the character reconstruction resulting from the accelerated transformation optimization (Maddison and Maddison 1992) is displayed.

crassifemur Grimshaw (subgenus *Engiscaptomyza*); at the other extreme, some species have filaments that are three or more than four times the length of the egg (Kambysellis and Heed 1971). Phylogenetic mapping of this egg trait, relative filament length, on the molecular tree (Figure 2*b*) shows a phylogenetic progression in Hawaiian drosophilids from no filaments to short filaments, to intermediate filaments, to extremely long filaments, although among the picture-wings there are some reversals of character state, some of which appear to be ecologically significant (see Figure 1). The most uncharacteristic species is *D. mulli* Perreira & Kaneshiro, which has only two very short filaments rather than the four long filaments of related species.

The substrate of this species is not known, but adults are always found on the lower surfaces of fronds of the native Hawaiian palm *Pritchardia.*

The importance of the variation in this egg trait can be appreciated by considering the variation in oviposition substrates (Figure 1) and the function of these egg appendages. Where eggs are inserted into the substrate, the posterior end is inserted first, and the respiratory filaments at the anterior end project above the surface of the substrate. In this position, they act as "snorkels," serving the respiratory exchange needs of the developing embryo (Margaritis 1983, Kambysellis 1993) in the oftentimes hostile and anaerobic conditions of the decaying substrate. Clearly, the thickness of the substrate and the depth to which eggs are inserted are factors affecting the length of the respiratory filaments. We surmise that evolution of these structures may have been driven by selection exerted by the oviposition substrate. Where oviposition is exophytic (that is, eggs are not inserted into the substrate but are simply dropped onto the surface of the plant tissue [either flowers or leaves]), as in the case of the scaptomyzoids, respiratory filaments are unnecessary. Just as eyes and pigments in cave organisms have been lost (Howarth 1993), so too these structures have become rudimentary or been lost entirely. In the groups that use decaying leaves, the egg is inserted at an angle in the leaf, which is quite a thin substrate. The egg is generally completely embedded in the plant tissue, with the anterior end very close to the surface of the leaf. The respiratory filaments in this case are generally of moderate length (Table 1).

In decaying stems, soft tissue is much closer to the substrate surface than in the case of decaying bark, and hence the eggs of stem-breeding species have only moderate-length filaments. In bark breeders, the ovipositor (the structure used for oviposition) is inserted through cracks in the thick cortical layer of the bark and the eggs are then released into the more pulpy material deep below. The respiratory filaments generally project above the bark surface and in these eggs are necessarily longer than in eggs that are inserted more shallowly into the substrate.

Morphological Variation in the Ovipositor

The ovipositor in *Drosophila* consists of a pair of sclerotized valves or vaginal plates joined anteroventrally by a narrow sclerotized bridge and dorsally by a membrane. At rest, the anterior end of the ovipositor is covered by the eighth abdominal tergite; during oviposition, muscles connected to the base of the ovipositor control its extrusion from the body. Each vaginal plate bears a distinctive array of sensilla or sensory bristles. Typically, the ovipositor tip bears a more prominent pair of sensilla extending from the ventral side. Presumably, these various sensilla serve as chemoreceptors and mechanoreceptors that function in substrate evaluation and host-plant recognition (Stoffolano and Yin 1987) as the female searches for a suitable oviposition site.

The ovipositors of Hawaiian drosophilids display an extraordinary range of variation in both size and shape, first indicated by the light microscopic observations of Throckmorton (1966). Among the more than 40 species for which we have made SEM observations thus far, ovipositor length varies more than 12-fold, ranging from 83 μm in one of the small scaptomyzoid species (Table 1, Figure 3) to more than 1 mm in a picture-winged fly (data not shown). It should be pointed out that ovipositor length is not entirely independent of body length, there being a general allometric constraint. Thus there is a tendency for small flies to have short ovipositors and large flies to have long ovipositors, but breeding substrate is an even more important factor. This can be seen by comparing thorax and ovipositor lengths of various species in Table 1.

Ovipositor shapes are also amazingly diverse (Figure 3). The shape variation among the 30 species that are included on the molecular tree can be classified into seven categories (see Materials and Methods). Besides the proportional length and width differences (see Table 1), the form of the ovipositor tip is quite distinctive for each of the shape categories recognized here. The question is, what is the functional significance of the ovipositor size and shape differences we observe among Hawaiian drosophilid species?

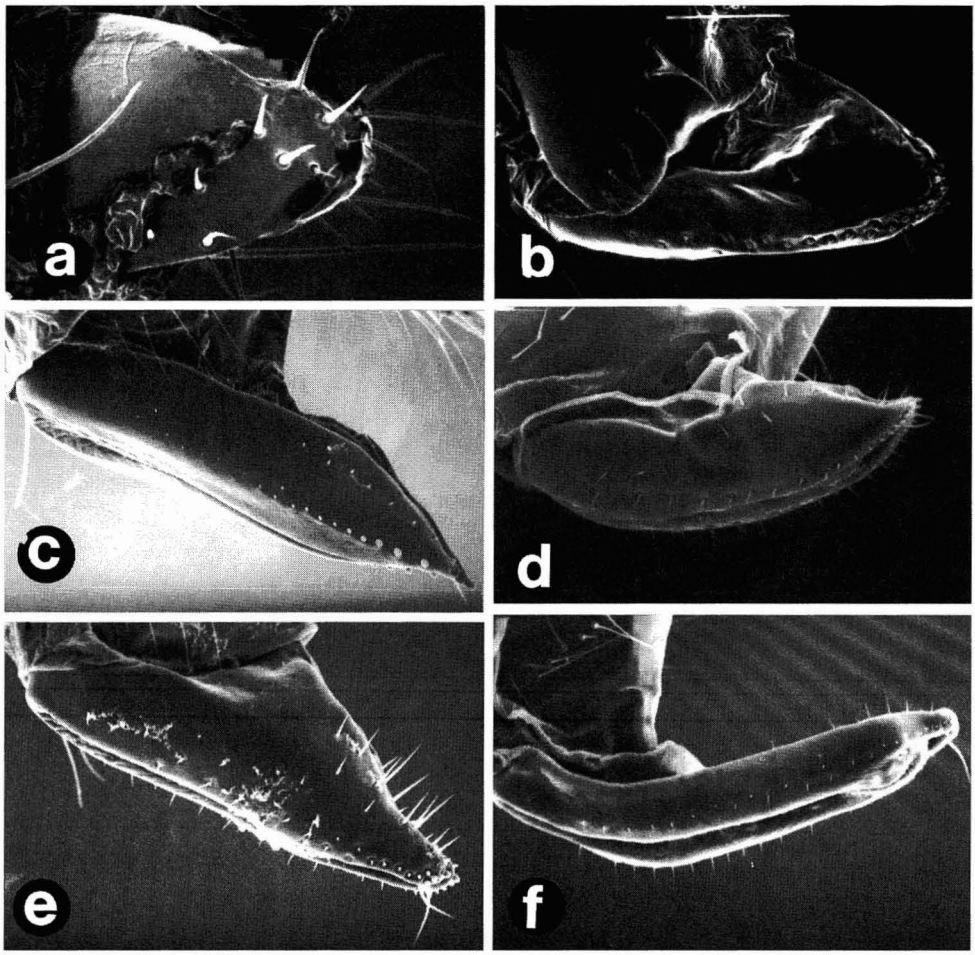

FIGURE 3. Scanning electron micrographs of Hawaiian drosophilid ovipositors, showing lateral views of species representative of six of the seven shape classes recognized here. The abdomen of the fly is to the left in each case. *a.* Small ovoid ovipositor of *Scaptomyza albovittata* (magnification 480 X); *b.* Oblong ovipositor of a fungus breeder, *D. longipedis* (mag. 197 X); *c. D. adunca* (a leaf breeder), showing the distinctive sharply pointed tip (mag. 89 X); *d. D. primaeva* (mag. 143 X); note the tip, which is somewhat pointed; *e.* Triangular ovipositor of *D. ornata,* a stem breeder (mag. 113 X); note the prominent spinelike sensilla on the dorsal side, in addition to the rows of sensilla typically found along the ventral edge of each ovipositor valve; *f.* Long, slender ovipositor of a bark breeder, *D. pullipes* (mag. 83 X).

Phylogenetic Pattern of Ovipositor Traits

Ovipositor length shows a general phylogenetic increase from short to long (Figure 4*a*), but this evolutionary trend is not entirely progressive, being confounded by differences in body size between sister clades. In particular, species of the relatively primitive antopocerus group are large flies (see thorax lengths of *D. adunca* (Hardy) and *D. diamphidiopoda* (Hardy) in Table 1) and have correspondingly long ovipositors (Figure 3*c*), much longer than those of their sister group, the smaller-bodied modified-tarsi group (represented in Table 1 and Figure 4*a* by *D. waddingtoni* (Basden)), and much longer than the ovipositors of the next most derived group, the modified-mouthparts group. Also, clades are not always homogeneous with respect to ovipositor length. To some extent, this may be a consequence of the fact that ovipositor

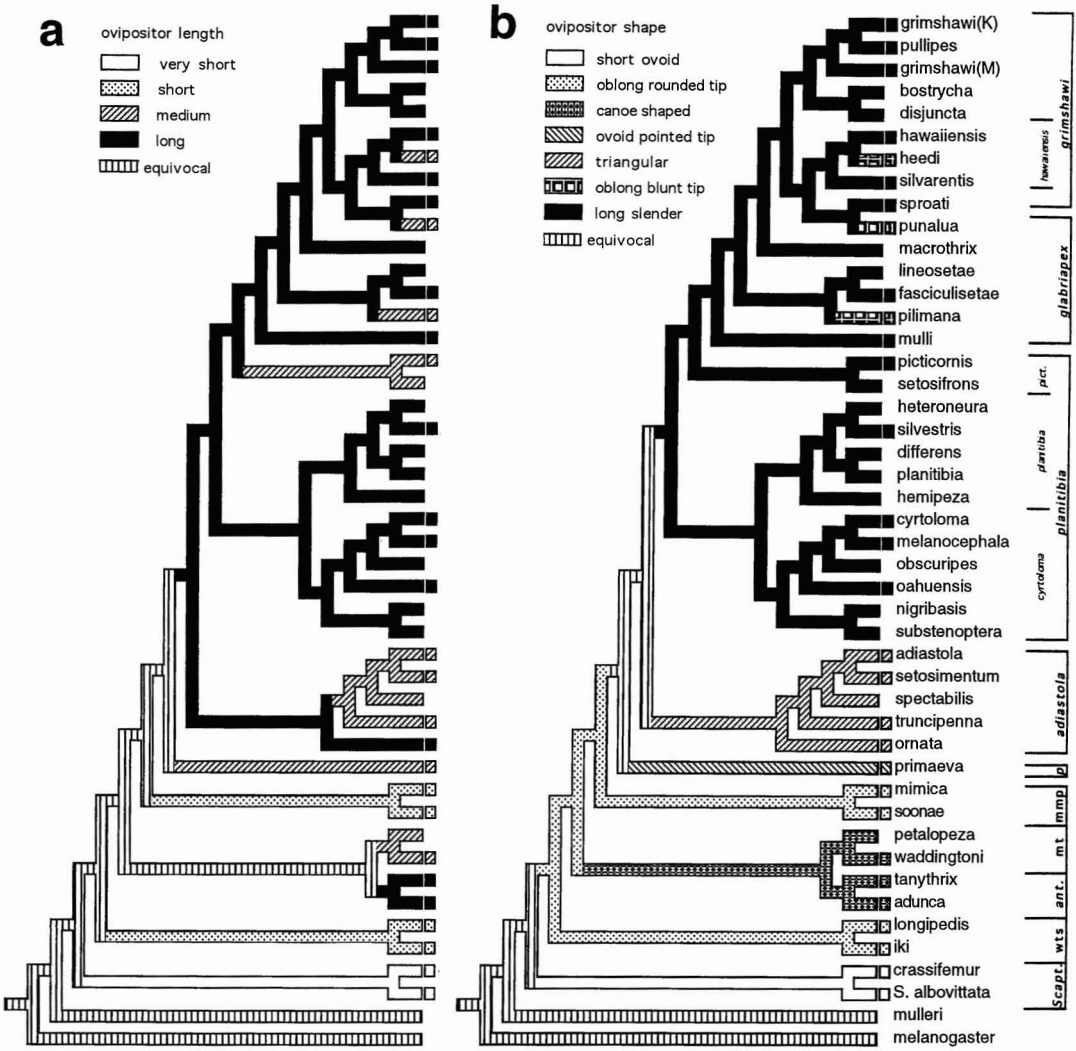

FIGURE 4. Evolutionary pattern of two ovipositor traits (*a,* length, and *b,* shape) among Hawaiian drosophilids. Measurements were made from the scanning electron micrographs (see Figure 3, and text for coding of character states). For both traits, the accelerated optimization is displayed. In the case of ovipositor shape, the patterns from the parsimony reconstruction and the accelerated transformation optimization were identical.

lengths have not been corrected for body lengths (the obvious next step in this analysis), and also the boundaries between size classes, chosen on the basis of natural breaks in the data set of 30 species analyzed here, may turn out to be somewhat arbitrary.

Comparison of the pattern of ovipositor length variation (Figure 4*a*) with the pattern of breeding substrate use (Figure 1) shows some but by no means perfect correspondence. Some of the discrepancies may result from missing ecological or ovipositor data (e.g., among the *planitibia* subgroup species of the *planitibia* species group). In other cases, the discrepancies are real and meaningful, as in the case of the flux-breeding *hawaiiensis* subgroup, where *D. heedi* has a shorter ovipositor than its sister species *D. hawaiiensis* Grimshaw and *D. silvarentis*

Hardy & Kaneshiro. Although generically grouped as a flux breeder, *D. heedi* females lay their eggs in flux drippings on the soil and never in the subcortical flux niche on the overhanging tree branches, which is used by the sympatric species *D. silvarentis.* In the adaptive shift from tree fluxes to soil fluxes, *D. heedi* has evolved a smaller body size, a shorter ovipositor (Figure 4*a*), and smaller eggs, with shorter respiratory filaments (Figure 2*b*) (Kambysellis and Craddock 1997).

Figure 4*b* shows the phylogenetic pattern of changes in ovipositor shape. In general, all the members of each clade have an ovipositor of the same characteristic form, but with substantial differences among lineages of Hawaiian drosophilids. It could be argued that this homogeneity within groups is due to phylogenetic and developmental constraints or, alternatively, that ovipositor shape is adaptive in terms of the function of this structure in depositing eggs in the variety of oviposition substrates. Because most groups are ecologically uniform (Figure 1), it might be predicted that ovipositor shape should be consistent within a lineage. Again, the exceptions are instructive. *Drosophila heedi* (discussed above) is ecologically distinct from the other members of the *hawaiiensis* clade, and its distinctive ovipositor shape is autapomorphic. *Drosophila punalua* Bryan is ecologically differentiated from *D. sproati* Hardy & Kaneshiro (Figure 1), and its ovipositor differs in length (Figure 4*a*) and in shape (Figure 4*b*) from that of *D. sproati.* These autapomorphies strongly suggest that ovipositor length and shape are candidates for adaptational explanations (Wanntorp et al. 1990). On the other hand, correspondence between the distribution of the phylogeny of the seven substrate classes recognized (Figure 1) and that of the seven ovipositor shapes (Figure 4*b*), although quite good, is not perfect. Specifically, the white-tip scutellum flies and the modified-mouthparts flies use quite different substrates—fungi, and fruit, respectively (along with stems and other substrates in the case of other members of the latter group)—yet their ovipositors are basically similar in size (Figure 4*a*) and in shape (Figure 4*b*). However, the species sampled may not be entirely representative of these two large and diverse groups, so any conclusions may be premature.

The distinctive elongated form of the ovipositor of the bark-breeding species (Figure 3*f*) is consistent for every species examined (including other bark-breeding species not included in the molecular phylogeny). The origin of this ovipositor form from the antecedent triangular ovipositor of the stem-breeding *adiastola* group species (Figure 3*e*) could most plausibly be interpreted as due to the action of strong selection on ovipositor length and shape characters, coincident with a shift from stem breeding to bark breeding. Whether selection has continued to operate to maintain long slender ovipositors in phylogenetically derived species is a moot point. It is noteworthy that the ovipositor form of *D. primaeva* (Figure 3*d*) is definitely not that of a bark breeder (Figure 3*f*), again indicating that the ecology of this species needs to be reinvestigated.

Historical Associations among Traits and Significance of Female Reproductive Variation

Phylogenetic tracing of changes in character states on an independent cladogram constructed using characters other than those under study (in our case molecular sequences) allows identification of apomorphic traits and visualization of the historical genesis of each particular trait. Comparisons of the historical patterns of evolution in several traits can identify coevolving traits, and, when considered in the context of the historical sequence of ecological shifts, suggest hypotheses concerning the forces responsible. The application of this approach to analyzing the remarkable female reproductive variation in the Hawaiian Drosophilidae provides some preliminary evidence that a common selective force may have acted on the female reproductive system of Hawaiian flies to drive the evolution of ovarian, egg, and ovipositor traits and thereby alter reproductive fitness. The environmental agent that is the most likely source of this selection is the breeding substrate. Colonization of the fungal substrate by the most primitive drosophiloid group, the white-tip scutellum flies, seems to have been associated with moderate increases in egg load and in the length of egg respiratory filaments and the ovipositor, compared with the condition of these traits in the more primitive scaptomyzoid flies. However, at

this level of the phylogeny, few firm statements can be made because the immediate ancestor of the Hawaiian flies is unknown, and the scaptomyzoids and white-tip scutellum flies are more likely related as sister groups, rather than as ancestor-descendant lineages. What we can more confidently assert is that the lack of respiratory filaments in scaptomyzoid eggs is an apomorphic trait resulting from the evolutionary loss of these structures, because the presence of respiratory filaments is characteristic of the eggs of all continental and all other Hawaiian drosophilids.

Colonization of decaying leaves seems to have been associated with a decrease in fecundity and evolution of a distinctively shaped ovipositor characterized by a sharp-pointed spine at the tip of each valve (Figure 3*c*). These spines may function in puncturing the leaf surface to facilitate the insertion of individual eggs in the substrate. The ovipositor traits and the reduced egg load are apomorphic for the whole clade of modified-tarsi and antopocerus flies and must have evolved in conjunction with adoption of the leaf-breeding habit.

In the most derived group of picture-winged flies, evolutionary patterns are more complex and this, together with some ambiguities in the reconstruction of ancestral character states, makes some of our interpretations tentative until more ecological and reproductive data can be gathered. Nonetheless, some events and trait associations are clear. Relative to the antecedent *D. primaeva,* evolution of stem breeding in the *adiastola* group has been accompanied by a substantial reduction in fertility (Figure 2*a*) and evolution of a distinctive triangular ovipositor (Figures 3*e,* 4*b*), although the respiratory filaments have remained at a moderate length (Figure 2*b*). The node leading to the *planitibia* group coincides with the transformation of the ovipositor to an elongate slender form (Figure 3*f,* 4*b*) with only three shape reversals above this node, and also the transition from moderate to extremely long egg respiratory filaments (Figure 2*b*). These two characters show parallel state transitions from the scaptomyzoids to the white-tip scutellum group and from the white-tip scutellum flies to the leaf-breeding antopocerus and modified-tarsi groups. Likewise, the three autapomorphic reversals in ovipositor form above the *planitibia* node correspond with three convergent reversals from long to moderate respiratory filaments (although the filament length reversal for *D. pilimana* Grimshaw involves the whole clade). The strong historical coincidence of state changes in these two traits, filament length and ovipositor form, suggests coevolution resulting from a common driving force, which might be hypothesized to be a change in selective regime caused by a shift in the breeding substrate. Such ecological shifts from stem to bark, with reversals from bark to stem, and in the case of *D. heedi* a shift from tree flux to soil flux, currently provide the most logical explanation for the observed patterns of character evolution and propose a series of hypotheses that are open to future tests.

The observed associations of ovarian traits with the nature of the ovipositional substrate (Kambysellis and Heed 1971), of ovipositor length and shape traits with the substrate (L. Franchi, P. Francisco, M.P.K., and E.M.C., unpubl. data), and the relationship between egg filament length and substrate type, as well as the historical patterns of coevolution in reproductive traits, all support the hypothesis that the female reproductive system of Hawaiian drosophilids is the target of selection exerted by the breeding substrate. Thus an ecological shift to a new substrate has often led to an evolutionary shift in one or more female reproductive traits, whether the ecological shift occurred early in the radiation of the Hawaiian drosophilids (Kambysellis et al. 1995) or comparatively more recently, as in the shift of *D. heedi* to breeding in flux drippings on the soil (Kambysellis and Craddock 1997).

By virtue of their vast number of species and remarkable diversity in form and in behavior, the endemic Hawaiian drosophilids compose what is widely recognized to be an evolutionarily successful group. One of the keys to their success must surely lie in the wide range of habitats that these flies have exploited for oviposition and larval development. Each species is generally monophagous, and females are highly selective in their oviposition behavior, generally choosing only one particular plant genus or family and only one particular part of the chosen plant in which to lay their eggs. Perkins (1913) quite astutely recognized the "tendency of island crea-

tures to . . . specialize their habits" or to adapt to their microenvironment; this adaptation to a highly specific habitat facilitated a reduction in interspecific competition and thus coexistence of multiple species. The diversification and adaptive radiation of the Hawaiian drosophilids is due in large measure to the fact that they have invaded and successfully exploited a wide variety of substrates for larval development. This exploitation has been facilitated by the lability of the female reproductive system—specifically, the evolution of a range of ovarian functional types containing anywhere from one to hundreds of mature eggs, the evolution of different types of eggs with short or long respiratory filaments or even no filaments at all, and the evolution of a wide variety of sizes and shapes of ovipositors that successfully insert eggs into the great diversity of substrates used by this group.

Phylogenetic analysis of these ovarian, egg, and ovipositor traits individually, and in combination, in the context of the historical pattern of ecological shifts, indicates that particular constellations of female reproductive traits have evolved in association with each specific substrate. It is hypothesized that natural selection exerted by the oviposition substrate has been a dominant force in molding these "egg-stage characters" into a diversity of reproductive outcomes. Our data on the eggs and ovipositors of Hawaiian *Drosophila* further corroborate the "insect egg" hypothesis proposed by Zeh et al. (1989), which posits that phyletic diversification is a function of the heterogeneity of habitats used for oviposition and larval development, and the suite of egg-stage characters that have allowed expansion into a series of novel niches. The Hawaiian Drosophilidae, with their remarkable species richness and concomitant diversity in female reproductive traits and oviposition sites, provide an outstanding example in support of this hypothesis and focus renewed attention on that remarkable device, the insect egg.

ACKNOWLEDGMENTS

Many of the scanning electron micrographs of the ovipositors of Hawaiian drosophilids were ably prepared by Lisa Franchi, some as part of her Senior Project at Purchase College, which was supported by a Texaco Undergraduate Research Award. We also thank Peter Francisco, another Purchase undergraduate, for preparing the initial SEM micrographs of ovipositors, Rob DeSalle of the American Museum of Natural History for sharing his phylogenetic data before publication, and two anonymous reviewers for their comments.

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