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Differences in venom composition between orb-weaving and wandering Hawaiian *Tetragnatha* (Araneae)

GRETA J. BINFORD*

Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA

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Spider venoms are complex mixtures of toxins that are used primarily for immobilizing prey. There is evidence of chemical variation in spider venoms among close relatives, yet few studies have analysed their evolution within an ecological and phylogenetic framework. On the Hawaiian archipelago, *Tetragnatha*, a cosmopolitan orb-weaving genus, has undergone a radiation in which a monophyletic lineage has abandoned web-building and become obligately wandering foragers. This study compares venom composition and details of feeding behaviour between orb-weaving and wandering Hawaiian *Tetragnatha*. Protein gel electrophoresis patterns indicated that relative to orb-weavers, wandering species had a reduced concentration of low molecular weight (<14 kDa) components. Both orb-weaving and wandering *Tetragnatha* captured flying prey (adult lepidopterans, dipterans), but wandering spiders also captured non-flying prey (insect larvae, spiders). There were no distinct differences between orb-weavers and wanderers in prey capture and immobilization sequences, or in the paralytic effects of bites on prey. However, prey bitten by wanderers took longer to be permanently immobilized than prey bitten by orb-weavers. Contrary to predictions, there was no indication that web-loss in this group was associated with an increase in venom potency.
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ADDITIONAL KEY WORDS: spider – venom – evolution – comparative – phylogeny – foraging – prey – diet breadth.

INTRODUCTION

Spider venoms are mixtures of peptides, proteins and polyamines that play a central role in immobilizing prey or, rarely, in self-defence. As in other venomous predators, there is substantial variation among taxa in the toxic mixtures making up venoms, and in their physiological effects (recent reviews – snakes: Tsetlin, 1999; cone snails: Olivera *et al.*, 1985; Craig, Bandyopadhyay & Olivera, 1999; Duda & Palumbi, 1999; scorpions: Possani *et al.*, 1999; spiders: McCormick & Meinwald, 1993; Atkinson & Wright, 1992; Olivera *et al.*, 1994; Shultz, 1997; Grishin, 1999; Escoubas, Diochot & Corzo, 2000). Discussions of the evolutionary processes influencing this diversity have largely focused on selection resulting from evolutionary change in feeding behaviour, and/or coevolution between venom toxins and their targets (Friedel & Nentwig, 1989; Nentwig, Friedel & Manhart, 1992; Olivera *et al.*, 1994; Daltry, Wüster & Thorpe, 1996; Le Gall *et al.*, 1999; Duda & Palumbi, 1999). However, no studies have used a phylogenetic comparative approach to integrate a study of patterns of change in venom composition with a study of patterns of change in the behavioral ecology of feeding.

Recent attention has focused on venoms for two reasons: they seem to undergo an unusually high rate of evolution at the molecular level (Olivera *et al.*, 1994; Duda & Palumbi, 1999); and they are a rich source of chemicals with novel structure and function (Jackson & Usherwood, 1988; Jackson & Parks, 1989; Adams & Olivera, 1994). As such, attempts have been made to predict venom characteristics based on aspects of the animal's biology (Friedel & Nentwig, 1989; Nentwig *et al.*, 1992). Across spider species, predation strategies vary widely and rely on venoms to varying degrees. Some spiders rapidly immobilize prey exclusively with venom (e.g. crab spiders, Thomisidae (Pollard, 1990)).

* Current address: Department of Biochemistry and Molecular Biophysics, University of Arizona, Tucson, AZ 85721, U.S.A. E-mail: gbinford@email.arizona.edu

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Others use silk for prey immobilization, and bite only after prey are completely enswathed in silk (e.g. some orb-weavers, Araneidae (Robinson, 1975)). Moreover, some spiders crush and chew prey early in the capture sequence, likely diminishing reliance on rapidly acting venom toxins. Ecological factors such as prey size, taxonomic category and risk potential, as well as the location of captures (in vegetation or on the ground) are also likely to affect the relative importance of venoms in prey immobilization (Friedel & Nentwig, 1989; Nentwig *et al.*, 1992; Boeve, 1994; Malli *et al.*, 1999).

This study analyses fine-scale evolutionary change in venoms among Hawaiian *Tetragnatha*, a lineage of spiders which has undergone an adaptive shift in feeding behaviour. Members of the cosmopolitan genus *Tetragnatha* typically capture prey using an orb-web. On the Hawaiian archipelago, *Tetragnatha* has undergone an evolutionary adaptive radiation in which a monophyletic lineage, the 'spiny leg clade', has abandoned web-building and adopted a wandering foraging mode (Gillespie, 1991a; Gillespie & Croom, 1995; Gillespie, Croom & Hasty, 1997). Both wandering and orb-weaving lineages have subsequently diversified in both morphology and microhabitat (Gillespie & Croom, 1995; Gillespie *et al.*, 1997; Gillespie, 1999). The availability of sympatric species that use different foraging strategies and the framework of a phylogenetic hypothesis (Gillespie, 1991a, 1999; Gillespie & Croom, 1995; Gillespie *et al.*, 1997) provide opportunities for comparing venom characteristics and details of feeding behavioral ecology among taxa that represent different times since divergence, and that have access to the same prey types yet use different strategies for prey capture.

The goals of this study are twofold: (1) to see if there is a difference between web-building and wandering Hawaiian *Tetragnatha* in venom chemical composition and/or the physiological effects of bites on prey; and (2) to identify detailed components of feeding behaviour and ecology that differ between orb-weavers and wanderers. Such differences are considered in the context of whether they infer a change in the functional role of venom in prey capture.

There are three possible outcomes of this comparison: (1) no differences between orb-weavers and wanderers in venom composition or the effects of bites on prey; (2) an increase in the immobilization effectiveness of wanderer venoms due to an increase in the relative amounts of components common to both lineages, or the origin of unique components; (3) a reduction in concentration, or loss, of particular components and a reduced immobilization effectiveness of bites in the wandering lineage. The *a priori* prediction was that bites of wanderers would more effectively immobilize prey than bites from orb-weaver to compensate for the loss of the role of silk in prey capture. This is based on general broad surveys of spider venom potency that suggest wanderers tend to have more potent, quickly acting venoms than web-building spiders (Friedel & Nentwig, 1989; Nentwig *et al.*, 1992). Although *Tetragnatha* webs are characteristically flimsy and function primarily to intercept prey while restraining only the smallest and weakest prey (Yoshida, 1987; Gillespie, pers. comm.; pers. obs.), the prediction was that the loss of the role of silk, or some other correlate of wandering would be reflected as an evolutionary change in venoms that increased the immobilization effectiveness of bites by wandering species relative to orb-weaving species.

METHODS

TAXA INCLUDED IN ANALYSES

Hawaiian Tetragnatha

Of the 34 currently described species, and roughly 60 'morphospecies' of *Tetragnatha* on the Hawaiian Islands (Gillespie, 1999), venom was sampled from females of 21 species (Fig. 1). All spiders were field collected in June and July, 1994, and May through July, 1996 by visually searching at night when the spiders are normally active. On Maui, collections were made at two sites on the windward (north) slope of east Maui. The following species were collected from The Nature Conservancy of Hawaii's Waikamoi Preserve: *T. quasimodo*, *T. waikamoi*, *T. kamakou* (Gillespie, 1991a), *T. tritubercula*, *T. eurychasma* near Carruther's Camp (1876 m); *T. stelarobusta*, *T. filiciphilia* (Gillespie, 1992a), and *T. brevignatha* (Gillespie, 1991a) from the Maile trail (1340 m).

On the island of Hawaii collections were made at two sites on two of the five volcanoes: *T. brevignatha*, *T. quasimodo* (Gillespie, 1991a), *T. perkinsi* and *T. hawaiiensis* (Simon, 1900; Okuma, 1988) were collected in Pu'u Maka'ala Natural Area Reserve (1200 m) on Mauna Loa; and the undescribed morphospecies referred to as 'elongate Hawaii' from the Maulua tract of the Hakalao National Wildlife Refuge (1610 m) on Mauna Kea.

On the island of Oahu in the Waiamae mountain range, *T. quasimodo* and *T. polychromata* (Gillespie, 1991a) were collected from Pahole Natural Area Reserve; and *T. perreirai* (Gillespie, 1991a), 'bicolor jaws', 'emerald ovoid', and 'elongate Ka'ala' were collected from the summit of Mt Ka'ala Natural Area Reserve (1220 m). In the Southern Koolaus mountains (427 m) *T. tantalus* (Gillespie, 1991a) and 'elongate Tantalus' were collected along a roadside on Mount Tantalus.

On the island of Kauai, *T. kauaiensis* and *T. pilosa*

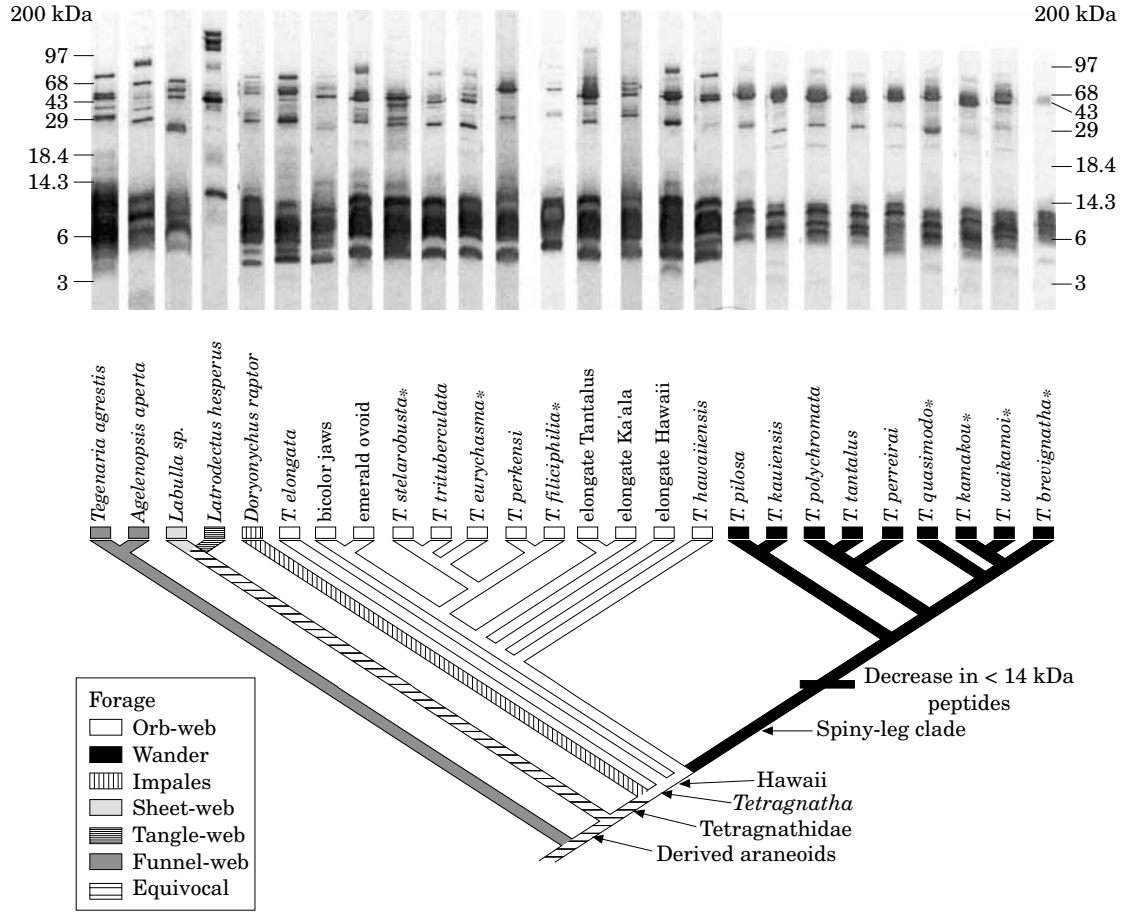

Figure 1. A composite phylogenetic hypothesis of the evolutionary relationships of the species included in this analysis based on Gillespie *et al.* (1997), Gillespie and Roderick (pers. comm.), and Scharff & Coddington (1997). Stars next to taxa indicate species for which behavioral data were collected. Electrophoretic banding patterns of TSDS-PAGE separations of proteins in whole venoms of female spiders are mapped at the tips of the phylogeny. Gel lanes are not precisely aligned by size. General size references are given at each end of the sequence of gel lanes. Foraging behaviour is reconstructed on the branches of the phylogeny.

were collected from Kokee State Park along the Pihea trail boardwalk (1220 m).

Outgroups

Venom was also sampled from six species outside of the Hawaiian *Tetragnatha* clade. Venoms of *Latrodectus hesperus*, *Agelenopsis aperta*, and *Tegenaria agrestis* were included to verify that electrophoretic patterns were consistent with known component sizes in well-characterized venoms. Venoms of *Tetragnatha versicolor* were included to polarize the direction of evolutionary change in the wandering lineage. Venoms of *Doryonychus raptor* were included because they represent an independent loss of web-building in Tetragnathidae on the Hawaiian Islands (Simon, 1900; Gillespie, 1991b, 1992b). Venoms were also included from a large, sheet-web building, endemic Hawaiian species (sympatric with Hawaiian *Tetragnatha*) from an undescribed genus of linyphiid that was formerly placed in '*Labulla*' (new species 2, Hormiga, pers. comm.).

T. versicolor (Walckenaer, 1802) was collected from Molino Basin in the Santa Catalina Mountains, Pima County, Arizona. *Doryonychus raptor* was collected from the Waiahuakua Valley on the Hono O Na Pali Natural Area Reserve (390 m) on Kauai. '*Labulla*' (new species 2) was collected from Pahole Natural Area Reserve on Oahu. *Latrodectus hesperus* was collected from a house in central Tucson, Pima County, Arizona. *Agelenopsis aperta* (Walckenaer, 1802) was collected from along Cave Creek in the Chiricahua Mountains, Cochise County, Arizona, near the American Museum of Natural History Southwestern Research Station;

and *Tegenaria agrestis* (Walckenaer, 1802) was collected from an abandoned shipyard in Marysville, Snohomish County, Washington.

Phylogenetic resolution among species included

The phylogenetic relationships used as the framework for comparisons of *Tetragnatha* species (Fig. 1) are based on ongoing analyses that currently provide sound support for the relationships among some species and ambiguity for others (Gillespie *et al.*, 1997; Gillespie, pers. comm.). The monophyly of the wandering 'spiny-leg' clade has been unambiguously supported in all analyses (Gillespie, Croom & Palumbi, 1994; Gillespie *et al.*, 1997; Gillespie, pers. comm.). The monophyly of the Hawaiian orb-weaving clade is not as clearly supported (Gillespie, pers. comm.). Species-level relationships within these clades are ambiguous and the phylogeny presented in Figure 1 represents a composite summary of the most recent analyses (Gillespie *et al.*, 1997; Gillespie, pers. comm.). In analyses using ribosomal 12S mtDNA, a mainland orb-weaving species, *T. pallescens*, falls within the Hawaiian species suggesting this radiation originated from more than one colonization event (Gillespie *et al.*, 1994). Thus, it is currently unclear if the spiny-leg clade is the immediate sister to the Hawaiian orb-weavers, and conclusions about the timing of changes are made with caution.

VENOM COLLECTION

Ontogenetic, seasonal, and intersexual variation in spider venom potency and/or chemical composition is known in some spiders (Atkinson & Walker, 1985; Malli, Vapenik & Nentwig, 1993; de Andrade *et al.*, 1999; Binford, 2001). Sources of variation were minimized by: (1) collecting within small geographic areas (single populations), (2) using only females collected as adults (with the exception of *L. hesperus* that were lab-reared adult female offspring of field-caught spiders), and (3) restricting the collecting period to June through August.

Spiders were anaesthetized with CO_2, and venom was extracted by electrical stimulation. To avoid contamination, fangs were rinsed with distilled water, and regurgitate was collected by a vacuum attached to a blunt syringe needle held on spider's mouths. Because *Tetragnatha* and *Doryonychus* produce very small quantities of venom, venom droplets from tips of their fangs were drawn into capillary tubes stretched to a fine point. Venom from other species was drawn into non-stretched 5 µl capillary tubes placed over the fangs. Venom from all species was stored in 0.1% acetic acid and frozen at −80 °C until analysis. Spiders were fed one day after milking, and were milked again four to seven days after feeding. The length of each spider from the dorsal, anterior tip of the cephalothorax to the posterior tip of the abdomen, was measured after milking.

To obtain sufficient quantities of venom for analyses, samples were pooled from adult females matched by species and population. The number of individual venom samples per pooled collection varied because of differences in the availability of spiders, attrition within groups, and occasional failure of a spider to yield venom. Although pooling masks variability at the individual level, pooled samples represent a population-level composite that is appropriate for investigating differences between populations and species. When there were sufficient numbers of individuals, multiple groups of individuals were pooled within each population. This enabled verification of consistency of chemical separation profiles within species.

CHEMICAL ANALYSES OF VENOMS

The approximate concentration of proteins in each sample was estimated by measuring absorbance at 280 nm using a Pharmacia Genequant spectrophotometer. The amount of protein yielded per spider was estimated by dividing total protein per sample by the number of individuals milked per sample.

SDS gel electrophoresis

Venom components were separated by tricine-sodium dodecyl sulfate (TSDS) polyacrylamide gel electrophoresis (PAGE) following the method of Schägger & von Jagow (1987). This method provided size information for components as small as 2 kDa. Unless otherwise noted, all analyses used approximately 35 µg of crude venom. Gels were stained with silver stain which has a detection limit of 2–5 ng per protein band (Silver Staining basic protocol, Current Protocols in Molecular Biology, 1999). All gels included 10 lanes with low range standards (2.75–43 kDa) on the far left lane and high range (14.3–200 kDa) on the far right lane, and were run using a tall, mini-gel apparatus ($9 \times 5 \times 0.1$ cm) (Hoeffer).

Gel quantification

Venom components separated by electrophoresis were analysed by estimating size and concentration of protein bands using BioImage Whole Band Analysis software. Sizes were estimated by comparing migration distances of bands to migration distances of standards of known size. Concentrations of protein bands were estimated by integrating their intensity. Percent integrated intensity was calculated for each protein band. Differences between orb-weavers and wanderers were quantified by comparing the average total percent

intensities of bands and groups of bands in different size regions. Percentages were arsine transformed before analyses. Data for venoms of *Tetragnatha versicolor* were included as an outgroup in quantitative analyses to help interpret the direction of evolutionary change.

A comparison of the presence and absence of particular proteins was difficult for two reasons: (1) small samples of venoms from some species did not allow multiple gel separations that would be necessary to detect components present in small amounts (the lack of visibility of a band did not necessarily mean the band was not present in a population); (2) it is impossible to know without further analysis if components that are the same size (migrate the same distance on the gel) are the same (homologous) polypeptides. For discussion, components of the same molecular weight across species of Hawaiian Tetragnatha were assumed to be homologous. This is reasonable based on parsimony arguments given the close relationships among these species. Components that were detected at least one time in a species were considered to be present even if they were not detected in all analyses of that species. Because of these difficulties analyses focus primarily on large-scale differences (between clades) and comparisons of the presence and absence of particular components are limited and interpreted with caution.

BEHAVIORAL ANALYSES

Behavioral analyses focused primarily on Hawaiian *Tetragnatha* species that live sympatrically on windward East Maui on The Nature Conservancy's Waikamoi Preserve (indicated on Fig. 1).

Taxonomic composition of natural prey

Samples of natural prey were collected directly from the chelicerae of any *Tetragnatha* found feeding in the field. Prey remains were taken to the laboratory and identified. Live specimens of the most numerous prey types were collected and preserved as voucher specimens and for measurements of body size.

Prey handling and immobilization

Details of prey capture and immobilization of different prey types by orb-weaving and wandering *Tetragnatha* species from windward East Maui (indicated on Fig. 1) were observed in the laboratory at The Nature Conservancy House at Haleakala National Park. These species were used because (1) they occur sympatrically and have access to the same prey types, (2) adults were locally abundant at the time of this study. Spiders were housed in 200 ml cups (9 cm diameter, 4.5 cm tall) that contained one centimetre of clean gravel and water to maintain humidity. Branches and leaves were placed in the cup to provide substrate for walking and silk attachment. Spiders were acclimated for at least 3 days with no food before prey were introduced and feeding behaviour was observed. Observations were conducted under a dissecting microscope in the lab while data were recorded on a data logger using a program created by Wayne Maddison (Hypercard). Prey were collected near the field station and included *Drosophila*, anthomyiid flies, other flies (Tipulidae, Sepsidae), and adult lepidopterans (Geometridae, Pyralidae). Body lengths of insect prey were recorded at the beginning of each observation, and lengths of spiders were recorded after each observation to minimize disturbance. The following details were recorded: (1) the general sequence of events involved in prey capture and immobilization; (2) duration of the initial bite (the time between which the spider first embedded its fangs in the prey and the time when the spider first started to chew the prey); (3) immobilization time (the time between when the fangs first entered the prey and the last prey movements were observed); and (4) the physical location of bites on the insect.

The effects of natural bites on prey

The effect of envenomation on prey in the absence of chewing was determined by allowing spiders to bite prey then removing prey from the jaws before the spider began to chew. Prey were then observed under a dissecting scope and the behaviour, physiological states of the prey (assessed by physical movement, response to prodding and visibility of a heartbeat), and the timing of changes in these states were recorded. In particular, the onset of the first obvious effect, the collapse time (time after which the animal lost its righting response) and the time of death (when there was no visible movement or heartbeat) were recorded relative to the time when the fangs first entered the prey. The timing of these events was compared between bites from orb-weavers and wanderers on anthymyiid and drosophilid prey. Although assessing venom effects by allowing spiders to bite prey and removing them confounds the effect of intoxication and the mechanical effect of impalement with fangs, and does not allow standardization for the amount of venom injected, it is a biologically realistic measure of the effect of natural bites on prey.

All analyses made use of the statistical software JMP (SAS).

RESULTS

VENOM COMPOSITION COMPARISON

Venom protein yielded per spider

The size-corrected yield of venom per spider (μg protein/milking/spider length (mm^3)) averaged across orb-weaving species was not significantly different from

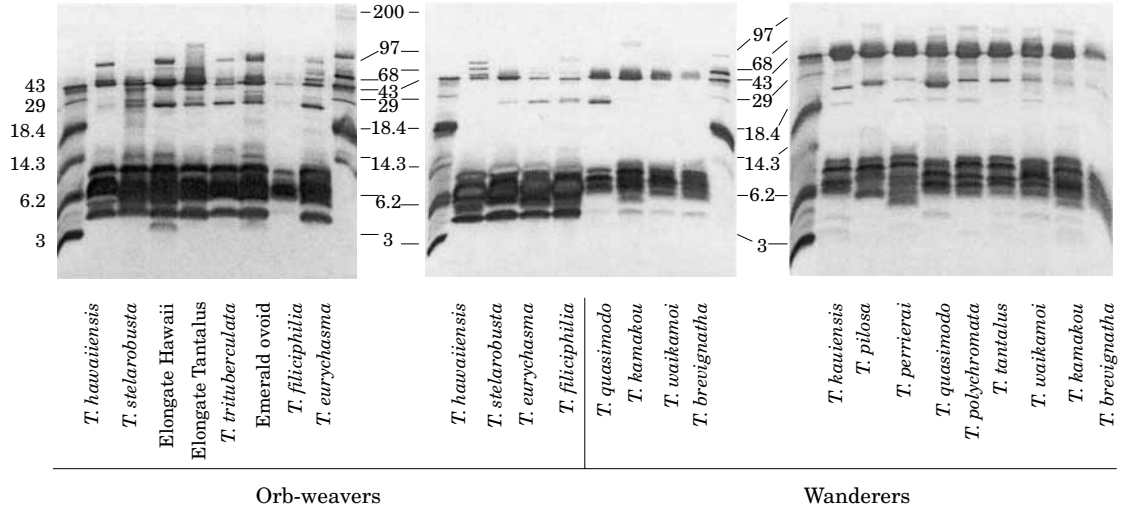

Figure 2. Examples of TSDS-PAGE electrophoretic separations of venoms of orb-weaving and wandering Hawaiian *Tetragnatha*.

the average across wandering species (each species weighted as 1) ($t_{70}=1.17$; $P=0.25$). Analyses of variability in size standardized protein yield per milking among species within the orb-weaving and wandering clades included only species for which three or more samples were available. There were significant differences among the wandering species *T. brevignatha*, *T. quasimodo*, and *T. waikamoi* ($F_{3,25}=6.43$, $P=0.003$). However, differences were not significant among the orb-weaving species *T. stelarobusta*, *T. filiciphilia*, *T. eurychasma*, *T. trituberculata*, *T. hawaiiensis* and elongate Hawaii ($F_{5,21}=2.17$, $P=0.097$).

General description of electrophoretic patterns of venoms

At least 26 individual proteins and peptides ranging in size from 3 kDa to 230 kDa were detected in crude venoms from samples across all Hawaiian *Tetragnatha* (Figs 1, 2). In all species and populations, the highest concentrations of components were between 3 kDa and 14 kDa. Individual bands were difficult to distinguish in this region, but there were at least 6 to 9 bands in some samples each likely containing multiple peptides. All *Tetragnatha* had at least one band around 25 kDa, a strongly staining band around 43 kDa, and a varying number of bands between 40 kDa and 90 kDa. Occasional bands were detected around 200 kDa. For comparative analyses the integrated intensities of bands within the size regions <14 kDa, 14–35 kDa, 35–80 kDa, and >80 kDa were combined (Table 1). These categories were selected to separate the most conspicuous banding regions.

Comparisons of banding patterns of venoms between different groups of *T. quasimodo* individuals, and among sequential milkings of the same groups of individuals yielded no obvious differences in electrophoretic patterns.

Table 1. Average and standard error of percent intensity of different size regions of proteins for orb-weaving and wandering Hawaiian *Tetragnatha*. MANOVA analyses were conducted on arcsine transformed data and excluded data for % >80 kDa. Below are results of the whole model MANOVA analysis including the feeding effect and effect of species nested by feeding type

Size range (kDa)	Orb-weavers	Wanderers	MANOVA effect test	
			F	*P*
% <14	80.7 (1.4)	69.3 (2.0)	$17.07_{1,46}$	<0.0001
% 14–35	8.3 (3.1)	5.8 (3.4)	$2.58_{1,46}$	0.1132
% 35–80	9.8 (0.8)	18.7 (1.1)	$7.10_{1,46}$	<0.0001
% >80	1.8 (1.1)	1.7 (0.8)		
		F	*P*	
Whole model		$2.85_{60,132}$	<0.0001	
Feeding		$22.26_{3,44}$	<0.0001	
Species [feeding]		$1.91_{57,132}$	<0.0013	

Comparison of electrophoretic patterns between orb-weavers and wanderers

A significantly larger amount of orb-weaver venom consisted of components <14 kDa as compared to wanderer venom (Table 1, Figs 1, 2). This was true for both absolute concentration ($t_{63}=5.02$, $P<0.001$) and

percentage total concentration (Table 1). The absolute concentration of components >14 kDa was not different between the orb-weavers and wanderers ($t_{63}=0.326$, $P=0.75$). Venoms of wandering species had higher percent concentrations (Table 1) and absolute concentration ($t_{63}=6.59$, $P<0.001$) of components between 35 and 80 kDa than did venoms of orb-weavers. However, the difference in absolute concentration did not persist when *T. quasimodo* data were excluded.

The phylogenetic relationships among species within the orb-weaving and wandering lineages present a statistical problem of nested levels of non-independence which is not addressed in this analysis. However, the difference in the concentration of components <14 kDa is clear and consistent (Figs 1, 2). Thus, the significance of this difference would likely not change using analyses that take into account phylogenetic relationships.

Three high molecular weight components were detected in low concentrations in orb-weaver venoms but not in wanderers. Detectability of these components was variable even within the same sample run on different gels. However, they were never observed in venoms of wanderers even on the same gels on which these components were detected in orb-weavers. These were a 230 kDa component (0.16 ± 0.05% of total concentration) (*T. stelarobusta*, *T. hawaiiensis*, elongate Hawaii, and emerald ovoid), a 214 kDa component (0.2% of total concentration) (*T. perkensi*), and a component(s) roughly 200–205 kDa (0.23 ± 0.12% of the total concentration) (*T. perkensi*, *T. stelarobusta*, *T. hawaiiensis*, elongate Ka'ala, *T. tritubercula*). Components of these sizes have also been detected in venoms of male *T. versicolor*, male Hawaiian orb-weavers, and male wanderers (unpublished data) and are therefore not truly unique to orb-weaving females. No components were detected that were unique to the wandering clade.

Variability within orb-weaving and wandering clades

There were some consistent differences between species within both the orb-weaving and wandering clades. Differences were detected both in the presence and absence of bands, and in the relative amounts of bands that were present in all species. No bands were unique to a particular species or clade. However, some bands were detected in diverse species. Venoms from both the wandering species *T. brevignatha* (Maui) and the orb-weaving species *T. stelarobusta* had a 150 kDa component that were not found in any other species. Also a 3 kDa band was detected in the North American orb-weaver *T. versicolor*, Hawaiian orb-weavers *T. stelarobusta*, elongate Hawaii, *T. tritubercula*, emerald ovoid, and *T. eurychasma*; and the wanderers *T. brevignatha* (Maui), *T. kauiensis*, *T. pilosa*, *T. perrierai*, *T. polychromata*, *T. tantalus*, and *T. kamakou*. Neither of these bands was detected in venoms from the wandering species *T. quasimodo* even though 7 independent samples were run for this species on a total of 16 different separations.

Within orb-weavers, species varied significantly in the percent total concentrations of components between 35 and 80 kDa ($F_{10,23}=4.72$, $P=0.001$). No other regions were significantly variable among orb-weavers. Within the wandering clade, species varied significantly in the concentration of components in all four size regions (<14 kDa, $F_{8,17}=5.39$, $P=0.002$; 14–35 kDa, $F_{8,17}=17.11$, $P=<0.001$; 35–80 kDa, $F_{8,17}=3.66$, $P=0.012$; >80 kDa, $F_{8,17}=3.23$, $P=0.02$). However, none of these regions was significant if data for *T. quasimodo* were excluded (<14 kDa, $P=0.219$; 14–35 kDa, $P=0.423$; 35–80 kDa, $P=0.214$; >80 kDa, $P=0.886$). Differences between pooled orb-weavers and wanderers in the percent total concentration <14 kDa and 35–80 kDa nevertheless persisted when *T. quasimodo* data were excluded from the comparison.

Significance of ANOVA tests of percent concentrations of gel regions within orb-weavers was not affected when data for the *Tetragnatha* outgroup species *T. versicolor* were included. Furthermore, the significance of comparisons between orb-weavers and wanderers of percent concentrations of gel regions were not affected by inclusion of *T. versicolor*.

Tetragnatha vs *outgroups*

General electrophoretic patterns of venoms from the Tetragnathidae *T. versicolor* and *Doryonychus raptor*, and the Linyphiidae '*Labulla*' closely resembled those for Hawaiian *Tetragnatha* (Fig. 1). The general size regions of bands were also similar between *Tetragnatha* and those for *A. aperta* and *T. agrestis*. However, banding patterns within these general regions were clearly distinctive relative to *Tetragnatha*. The general size regions of components in *Latrodectus hesperus* venoms differed from all other samples (Fig. 1). All of the venoms from outgroups except '*Labulla*' and *D. raptor* are described in detail elsewhere (see Discussion).

TAXONOMIC COMPOSITION OF NATURAL PREY

Samples of prey and the spider species from which they were collected are summarized in Table 2. Because *Tetragnatha* chew prey during consumption, accurate taxonomic identification was not possible in all cases. Consequently, data were summarized at the level of accuracy at which there was consistent feasible identification.

Overall, diets consisted of prey from a range of arthropod orders. However, wandering *Tetragnatha* consumed a wider range of taxa than orb-weavers.

Table 2. Percentages of different types of prey collected from the chelicerae of Hawaiian *Tetragnatha* in May–July of 1994 and 1996. Numbers in parentheses are sample sizes

	PREY										
	FLYING					CURSORIAL					
	Lepidoptera	Diptera		Homoptera	Psocoptera	Lepidoptera larvae	Diptera larvae	Coleoptera larvae	Araneae		Amphipods
		Tipulidae	Other dipterans						Other spiders	*Tetragnatha*	
ORB-WEAVERS											
T. stelarobusta (40)	78	12		5	5						
T. filiciphilia (21)	14	5	67	5			5		5		
T. eurychasma (42)	7	10	67	10	2				2	2	
Elongate Hawaii (8)	88		12								
T. hawaiiensis (6)	33		17	50							
Bicolor jaws (2)											100
WANDERERS											
Maui											
T. quasimodo (74)	14	6	6	11		40	7	7	4	6	
T. kamakou (36)	11	9	20	28		22	3	6	3	3	
T. waikamoi (35)	9	6	37	14	12	14			3	3	
T. brevignatha (3)		33		33						33	
Big Island											
T. quasimodo (8)				25					25	50	
T. brevignatha (3)	33			67							

Wanderer diets included both flying and substrate bound prey, whereas orb-weavers were found eating predominantly flying prey. An exception is the orb-weaver 'bicolor jaws', individuals of which were found consuming only terrestrial amphipods. Other spiders (including conspecifics and congeners), were more common in, but not unique to, wanderer diets. Larvae were frequently collected from wanderers, whereas only in one case was a larval dipteran collected from an orb-weaver. The diets of orb-weavers included only nocturnally active prey, whereas diets of wanderers included prey that roost at night.

PREY CAPTURE AND IMMOBILIZATION

Prey capture sequence

The general sequence of events during prey capture and immobilization was the same for all species of orb-weavers ($N=45$) and wanderers ($N=69$). It was also similar across captures of different prey types. In all cases, once prey were physically encountered, they were seized in the spider's chelicerae and held ('initial bite'). No wrapping with silk was observed during the immobilization phase. After some period of time the spider would move its chelicerae, moving the fangs in and out of the prey and crushing the prey between the fang and the cheliceral teeth ('chewing'). Both during the initial bite and after chewing started, the cephalothorax visibly moved up and down. This presumably corresponded with regurgitation of digestive enzymes and perhaps the action of the sucking stomach.

For all prey types, initial bites were more frequently delivered to the thorax than to any other body segment (Fig. 3; 65% of 119 prey capture observations, 8% were to the thorax and at least one other body region). The tendency to bite the thorax did not significantly differ among spider species ($\chi^2=29.15$, $P=0.51$), among captures of different prey types ($\chi^2=22.85$, $P=0.30$), or among species grouped by foraging type ($\chi^2=4.05$, $P=0.54$).

Timing of prey capture sequence

For all six species observed, the length of the initial bite did not vary significantly as a function of prey type (Table 3). There was a significant effect of spider species on initial bite times and immobilization times for captures of lepidopteran adults, but not for any other prey category. In the case of lepidopteran adults, neither differences in initial bites times, nor immobilization times were influenced as a function of prey ensnarement strategy (Table 3).

Both initial bite times and immobilization times were longer for captures of drosophilid prey by wanderers than for captures by orb-weavers (Table 3).

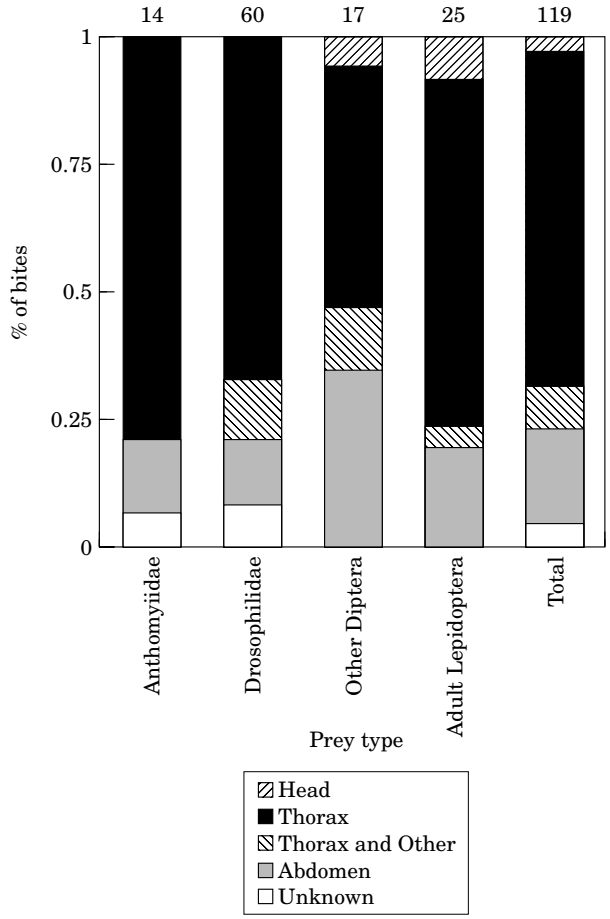

Figure 3. The location of the initial bite pooled across all *Tetragnatha* species for captures of different prey types.

Initial bite times and immobilization times did not differ between orb-weavers and wanderers for captures of any other prey type (Table 3), or for captures of all prey types pooled (initial bite, orb-weavers 26.5 ± 8.7, wanderers 30.8 ± 5.8 ($t_{113} = 0.41$, $P = 0.68$); immobilization time, orb-weavers 249.2 ± 93.9, wanderers 349.2 ± 84.2 (unequal variance (Welch); $t_{107} = 0.97$, $P = 0.39$). Significance of comparisons of pooled data was not affected when data for captures of Lepidoptera were excluded. The standard error of estimates of immobilization times for all captures combined was significantly higher for wanderers than for orb-weavers (Bartlett: $F = 16.64$, $P < 0.0001$).

Effect of venom on prey after natural bites

Anthomyiid and drosophilid prey removed from spider's jaws after the initial bite but before chewing were either paralysed immediately after removal, or exhibited a stereotyped series of physiological effects that did not differ between bites from orb-weavers or wanderers. The first visible effect was dragging the 2nd and 3rd pairs of legs. Then prey became motionless and if pushed over could not right itself. Prey then remained paralysed for some period of time during which there was a visible heartbeat and occasional movements of the mouthparts and ovipositor. Wings never flapped after the initial bite. No prey recovered after being bitten.

Average prey/spider size and timing of events of prey paralysis did not differ between anthomyiid and drosophilid prey. Therefore data for these prey types were analysed together. There were no differences between orb-weavers and wanderers in the length of time spiders bit anthomyiid and drosophilid prey before prey removal ($t_{14} = 0.40$, $P = 0.69$). Differences between foraging modes in the time between fang entry and the first visible effect were not significant ($t_{14} = 0.341$, $P = 0.74$) (Fig. 4). The time until collapse was marginally longer ($t_{14} = 1.86$, $P = 0.08$), and time until death significantly longer (unequal variance Bartlett $F = 3.98$, $P = 0.05$; Welch $t_{14} = 2.51$, $P = 0.05$) after bites from wanderers than from orb-weavers (Fig. 4).

DISCUSSION

This study identified a difference between orb-weaving and wandering Hawaiian *Tetragnatha* spiders in the concentration of chemical components <14 kDa in female venoms. There were also differences between orb-weavers and wanderers in the concentration of components between 35 and 80 kDa. The latter region, however, varied within orb-weaving and wandering clades and, thus, differences were not strictly associated with generalized differences in feeding behaviour. The lesser amount of low molecular weight components did not confer a difference in the qualitative physiological effects of bites on prey. However, it was correlated with a longer time until prey death. Also, wandering Hawaiian *Tetragnatha* consume a broader taxonomic range of prey than orb-weavers. There were no conspicuous differences between orb-weavers and wanderers in the way spiders immobilized prey or in the location of the initial bite on prey.

Quantitative and qualitative similarities between venoms of *T. versicolor* (the North American congeneric outgroup) and Hawaiian orb-weavers are evidence that the evolutionary change in concentration of low molecular weight components was in the direction of a reduction of components in this region in the wandering lineage as opposed to an increase in the Hawaiian orb-weaving lineage (Fig. 1). The change in venom composition in the wandering lineage represents a single evolutionary event. The loss of low molecular weight components was not repeated in

Table 3. Average, standard error and sample sizes (below) of (A) initial bite times (s) and (B) immobilization times (s) for laboratory captures of different types of prey by different *Tetragnatha* species. *P* values are from ANOVA using prey type (last column) or spider species (bottom rows in each section) as the grouping variable. Cases where there were only two prey types (*T. brevignatha*) are analysed using *t*-tests. 'W' indicates cases where variances were unequal (Bartlett test) and data were analysed using Welch ANOVA or *t*-test for unequal variances. Significant *P* values are in bold

Species	Size (mm)	Anthomyiid	Drosophilid	Other diptera	Lepidoptera adults	*P* value (ANOVA)
Size (mm)		4.6±0.4 14	2.1±0.8 60	4.8±1.7 19	7.25±1.8 25	
(A)						
INITIAL BITE						
T. eurychasma orb	4.6±0.7 18	117.7±93.6 3	14.0±2.7 9	61.0±60.5 3	49.5±35.5 2	0.558 (W)
T. filiciphilia orb	4.0±0.2 25	6.0 1	11.3±2.4 12	12.5±3.5 5	7.7±0.2 6	0.537
T. brevignatha wanderer	5.7±0.5 12	39.0±16 2	20.7±5.1 9			0.186
T. quasimodo wanderer	5.9±0.7 22	56.2±30.0 2	31.4±11.3 11	34.0±19.0 2	18.7±2.7 6	0.546
T. kamakou wanderer	4.9±0.7 11	17.0 1	18.8±5.6 5	36.5±5.5 2	314.0 1	0.130
T. waikamoi wanderer	6.6±0.5 30	12.2±2.1 5	17.7±6.0 12	4.7±3.3 3	26.6±6.5 8	0.078 (W)
P values (ANOVA)		0.431 (W)	0.306	0.567 (W)	**0.011** (W)	
Orb *vs* wanderer *t*-test		0.182	**0.022** (W)	0.723	0.6112	
(B)						
IMMOBILIZATION						
T. eurychasma orb	4.6±0.7 18	545.7±317.9 3	81.8±20.5 9	108.7±96.7 3	685.5±178.0 2	0.505 (W)
T. filiciphilia orb	4.0±0.2 25	30.0 1	88.1±16 12	125.3±72.1 6	782.7±298.6 5	**0.003**
T. brevignatha wanderer	5.7±0.5 12	221.5±85.5 2	366.0±175.2 8			0.689
T. quasimodo wanderer	5.9±0.7 22	1566.5±1549.5 2	137.4±47.9 11	26.0±21.0 2	170.5±45.9 4	**0.037**
T. kamakou wanderer	4.9±0.7 11	25.1 1	199.5±127.3 4	44.5±34.5 2	3168.0 1	0.465
T. waikamoi wanderer	6.6±0.5 30	138.6±61.9 5	112.6±34.8 11	4.0±2.3 3	877.3±318.0 7	**0.011** (W)
P values (ANOVA)		0.288 (W)	0.091	0.807	**0.030**	
Orb *vs* wanderer *t*-test		0.981	**0.037** (W)	0.175 (W)	0.757	

Doryonychus raptor, a tetragnathid species that represents an independent evolutionary loss of web-building (Gillespie, 1991b; Gillespie *et al.*, 1994, 1997) from what was likely similar ancestral behaviour. Therefore, inferring a causal link between the change in venom composition and change in other characters related to prey capture must be done with caution.

The lower concentration of low molecular weight components in wanderers relative to orb-weavers, and the apparent lower potency were counter to *a priori* predictions based on two previously observed patterns. First, comparisons of a few unrelated spider species suggested a general trend towards more potent venoms in non-web-building spiders (Freidel & Nentwig, 1989; Nentwig *et al.*, 1992) which seems reasonable given the loss of the restraining effects of prey entanglement in web silk. Although this analysis only represents one evolutionary event, the result falsifies a consistent simple rule of an increase in venom components or the immobilizing effects of bites when wandering evolves.

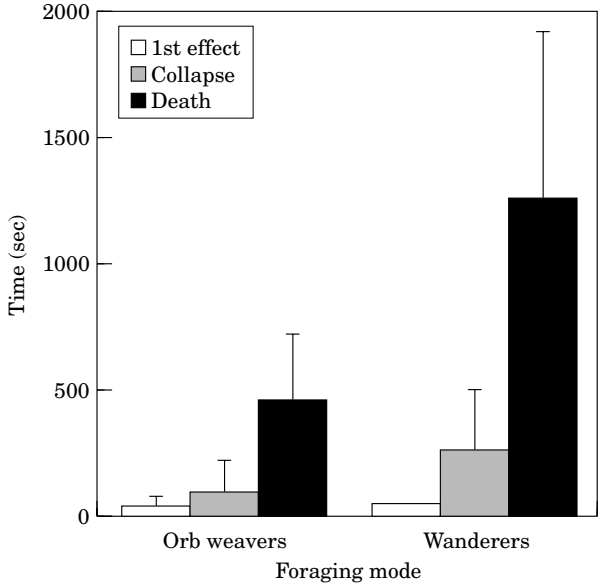

Figure 4. The average and standard deviation of times between when the fangs first enter prey and the first visible effect of venom, prey collapse, and prey death after prey were bitten then removed from the jaws of orb-weavers or wanderers.

Second, recent work investigating interpopulation variability in snake venom chemistry suggested that variation in diet correlated with differences in venom composition more closely than either phylogenetic or geographic distance in *Calloselasma rhodostoma* (Daltry *et al.*, 1996). Thus, at least in snakes, venoms may closely track ecological variability (but see Williams *et al.*, 1988; Sasa, 1999). If this were true in spiders, a gain rather than a loss of components might be expected in association with an expansion of the taxonomic range of prey consumed.

The counterintuitive (at least to the author) direction of evolutionary change in venom chemistry, and the lack of a convergent change in venoms of *D. raptor* is likely indicative of multiple parameters that influence venom characteristics. Therefore, predictions of general broad-scale patterns of venom characteristics may be difficult to support. Here, the patterns observed in this study are discussed in the context of potential influences of (1) diet breadth and (2) the historical legacy of prey interception and prey immobilization in the *Tetragnatha* lineage.

DOES DIET BREADTH CORRELATE WITH VENOM COMPOSITION?

If there was a direct causal link between the evolutionary change in venom chemistry and the loss of web-use in Hawaiian *Tetragnatha*, the most conspicuous detail of feeding ecology that might have effected this change was the difference in diet breadth between orb-weavers and wanderers. Wandering *Tetragnatha* generally consume a broader taxonomic range of prey that includes a smaller proportion of flying prey than orb-weavers (Gillespie & Croom, 1995; Gillespie *et al.*, 1997; Table 2). The evolutionary reduction of low molecular weight components in wanderers could be explained, for instance, if they are particularly effective for immobilizing flying prey that are consumed in lesser proportions than in the orb-weaving ancestors of this group. Comparative bioassays of low molecular weight components on different prey types would clarify whether this was the case.

A test of the influence of diet on venom composition is to compare venoms of specialist species to those of generalist relatives. There is some tendency towards prey specialization in many orb-weaving Hawaiian *Tetragnatha* that is likely due to differences in microhabitats of web placement (Gillespie & Croom, 1995; Gillespie *et al.*, 1997; Table 2). If venom variability closely tracks the taxonomic composition of prey consumed, one would predict more differences among orb-weaver venoms than among wanderer venoms. This low-resolution analysis gave no indication that venoms were more variable among orb-weaver than among wanderers. More detailed analyses of venom composition are necessary to answer these questions.

UNDERSTANDING VENOM CHARACTERISTICS AS A FUNCTION OF EVOLUTIONARY HISTORY

In addition to particularities of ecology and behaviour, the evolutionary history of a lineage may lead to preadaptation for different degrees of reliance on venoms for prey immobilization. This likely affects the degree to which venom composition changes in association with ecological or behavioral change. *Tetragnatha* generally make flimsy webs that trap prey but do not effectively aid in subduing most large prey (Yoshida, 1987). Thus, wanderers may be preadapted to rely on powerful jaws and venom for subduing prey, and the loss of web-use may have been of little consequence for prey immobilization. The greater standard error in immobilization times during captures by wanderers relative to orb-weavers, and the anecdotal observation that some prey actively struggled long after the initial bite (many hours), leads to the suspicion that wandering *Tetragnatha* have compensated for the capture of more diverse prey types by relying on the mechanical action of crushing prey to a greater extent than orb-weavers. If true, this implies that wanderers facultatively use venom to subdue prey perhaps depending on prey characteristics. Recent work on

Cupiennius salei shows that at least some spiders can facultatively regulate the volume of venom injected as a function of prey size and activity level (Boeve *et al.*, 1995; Malli *et al.*, 1999).

The loss of web use and increase in taxonomic range of prey available to the spiny-leg clade were not associated with a major shift in prey handling and immobilization tactics. All *Tetragnatha* seized prey in their jaws and did not wrap during initial stages of prey capture. Grabbing prey in chelicerae as the first attack is the ancestral condition within spiders, but attacking by wrapping first is found sporadically among araneomorph spiders and is thought to be advantageous for captures of risky prey (Eberhard, 1967; Robinson, 1969). Members of the tetragnathid genus *Nephila*, however, have always been observed to bite prey as the first attack independently of prey type (Robinson, Mirick & Turner, 1969). These behaviours appear to be conserved within Tetragnathidae, whereas the concentration of low molecular weight components in venoms, and the taxa of prey consumed do change in association with the loss of web-building. While behaviour is often discussed as being more evolutionarily plastic than other characters (Urbani, 1989; Burghardt & Gittleman, 1990; but see deQuieroz & Wimberger, 1991), in this case there is more stasis in behavioral characters involved in prey handling than in venom chemistry.

Finally, there are at least two possible explanations of the observed pattern that do not infer a direct causal link between the difference in venom composition and differences in feeding ecology. The general trend of more potent venoms in non-web-building spiders (Freidel & Nentwig, 1989; Nentwig *et al.*, 1992) could be broadly true, but the evolutionarily recent origin of wandering in Hawaiian *Tetragnatha* might make them unlike lineages that have been wandering for longer periods of time. They may be 'derived orb-weavers' as opposed to true wanderers. Alternatively, because wandering *Tetragnatha* represent an island radiation, perhaps they have undergone an ecological release that enables them to get by with less effective venom that is good enough in the relatively uncompetitive, un-coevolved community.

GENERAL VENOM DESCRIPTION AND COMPARISON AMONG OUTGROUPS

Evolutionary patterns of variability among species included in this analysis other than *Tetragnatha* were not interpretable because the phylogenetic resolution provided by these highly divergent taxa is inappropriate for deciphering patterns of change.

Although the chemical analyses in this study do not provide sufficient information for characterization of venom components, the patterns of sizes of proteins and peptides are consistent with general functional classes of toxins that have been described in spider venoms, many of these from species included here as outgroups. Polypeptide toxins (between 2 and 16 kDa) are common in spider venoms and typically interact with specific types of ion channels on excitable membranes (Adams, Herold & Venema, 1989; McCormick & Meinwald, 1993; Grishin, 1999; Escoubas, Diochot & Corzo, 2000). These are well described from the venoms of *Agelenopsis aperta* (Fig. 1).

Large proteins (100–150 kDa) known from *Latrodectus* typically bind to presynaptic neuronal membranes and stimulate massive neurotransmitter release (Finklestein, Rubin & Tzeng, 1976; review in Grishin, 1999). The bands that likely correspond to latrotoxins are visible in electrophoretic profiles of venoms of *L. hesperus* (Fig. 1). There were faint bands in this region in some *Tetragnatha* (Fig. 1), but the banding pattern for *L. hesperus* was strikingly different from all other taxa in the analysis.

Polyamine toxins (<1000 kDa) (reviews in McCormick & Meinwald, 1993; Shultz, 1997) are known from numerous, phylogenetically broadly distributed spiders, including *Agelenopsis aperta* (Skinner *et al.*, 1989) and tetragnathid species in the genus *Nephila* (Aramaki *et al.*, 1986). Faint staining was visible in this size region for *A. aperta*, but neither electrophoretic profiles nor unpublished HPLC data provided any hint of such toxins in the venoms of *Tetragnatha* (Fig. 1).

Physiological effects of bites from Hawaiian *Tetragnatha* show similarities to the effects of venoms from their closest relatives that have been characterized. Venoms from a variety of araneid spiders and *Nephila* block transmission at insect excitatory neuromuscular junctions (Kawai, Niwa & Abe, 1982; Usherwood, Duce & Boden, 1984; Usherwood & Duce, 1985; Jackson & Usherwood, 1988; Kawai, 1991). The visible effect on insects of venoms from the araneid *Argiope bruennichi* is a slow onset of paralysis progressing from the prothorax to the mesothorax that is amplified by locomotion and reversible (Friedel & Nentwig, 1989). This progression of paralysis is similar to that observed in prey after *Tetragnatha* bites, but in contrast to *A. bruennichi* the effects of Hawaiian *Tetragnatha* bites were irreversible on the time scale of these observations (2 to 3 hours). Prey that were instantly paralysed may have been affected by mechanical damage resulting from penetration of the fangs. It is notable that wing flapping was never observed after *Tetragnatha* bites. It cannot be determined whether this was the result of mechanical damage by the fangs or chemical inhibition of musculature. Nevertheless, it is likely advantageous in reducing the escape potential of flying prey and could partially

explain the preferential occurrence of bites to the thorax.

EVOLUTION OF VENOMS

In summary, this study revealed that associated with the loss of web use in Hawaiian *Tetragnatha* was a change in the relative amounts of components of lower molecular weights, some evidence of a reduction in the immobilization effectiveness of bites, and an expansion of prey types consumed. However, no change was observed in the general sequence of physiological effects of venom on prey or the role of venom in prey capture.

Two evolutionary processes that potentially underly venom diversification are selection due to changes in ecology or behaviour, and coevolution between toxins and their target receptors (Friedel & Nentwig, 1989; Nentwig *et al.*, 1992; Daltry *et al.*, 1996; Le Gall *et al.*, 1999; Duda & Palumbi, 1999). The data presented here lend support to changes in ecology underlying changes in venom composition. These data are also worth considering within the context of coevolution. Frequent origin of novel toxins or repeated evolution of the same toxins would be evidence of venom diversification driven by a coevolutionary arms race. The lack of detection of conspicuous components unique to clades or species may indicate that the origin of novel components is rare at the evolutionary time scale of the radiation of Hawaiian *Tetragnatha*. More detailed molecular analyses of particular toxins might reveal differences that were not detected by comparing whole venom composition, and are necessary to fully address this issue. However, the lack of conspicuous change in the general physiological effect of bites on prey indicates that, if there were underlying molecular differences in toxins that were not detected by electrophoresis, and these did have a consequential effect on target receptors, these differences were not visible in the general effects of bites on prey.

Given that individual venoms include many toxins, that most spiders consume more than one type of prey, and that most prey are consumed by more than one spider species, selection intensities for any coevolutionary processes underlying venom diversification in this lineage would likely not be strong (Thompson, 1999). If coevolution is not a major force driving venom diversification in generalist foragers such as spiders, a more feasible means of diversification might be bursts of change associated with shifts in the target animal or the ecology of prey capture, interspersed with periods of relative stasis. Detailed molecular analyses of well-documented evolutionary events that involve ecological shifts, such as that in Hawaiian *Tetragnatha*, would go a long way towards answering such questions.

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