Sexual dimorphism in venom chemistry in *Tetragnatha* spiders is not easily explained by adult niche differences

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ABSTRACT

Spider venom composition typically differs between sexes. This pattern is anecdotally thought to reflect differences in adult feeding biology. We used a phylogenetic approach to compare intersexual venom dimorphism between species that differ in adult niche dimorphism. Male and female venoms were compared within and between related species of Hawaiian *Tetragnatha*, a mainland congener, and outgroups. In some species of Hawaiian *Tetragnatha* adult females spin orb-webs and adult males capture prey while wandering, while in other species both males and females capture prey by wandering. We predicted that, if venom sexual dimorphism is primarily explained by differences in adult feeding biology, species in which both sexes forage by wandering would have monomorphic venoms or venoms with reduced dimorphism relative to species with different adult feeding biology. However, we found striking sexual dimorphism in venoms of both wandering and orb-weaving *Tetragnatha* species with males having high molecular weight components in their venoms that were absent in females, and a reduced concentration of low molecular weight components relative to females. Intersexual differences in venom composition within *Tetragnatha* were significantly larger than in non-*Tetragnatha* species. Diet composition was not different between sexes. This striking venom dimorphism is not easily explained by differences in feeding ecology or behavior. Rather, we hypothesize that the dimorphism reflects male-specific components that play a role in mating biology possibly in sexual stimulation, nuptial gifts and/or mate recognition.

Key Words: comparative, intersexual, Hawaii, phylogeny, mating, feeding

1 INTRODUCTION

2 Sexual dimorphism can be found in many traits of organisms, including conspicuous
3 morphological and behavioral phenotypes, and less visible biochemical and physiological
4 phenotypes. In spiders, differences between sexes in venom composition and/or potency have
5 been recognized in all species that have been studied (Atkinson 1981; Kent et al., 1984; Atkinson
6 & Walker, 1985; Friedel & Nentwig, 1989; Malli, et al., 1993; Celerier et al., 1993; Binford,
7 2001a; Escoubas et al., 2002; de Oliveira et al., 2005; Herzig et al., 2002; Escoubas et al., 2006;
8 Herzig et al., 2008; Herzig & Hodgson, 2009). Spider venoms are complex mixtures of toxins
9 that typically include peptides, polypeptides and polyamines (reviews in Kuhn-Nentwig et al.,
10 2011; Smith et al., 2013). Chemically, sexual differences can result from components that are
11 uniquely expressed in either sex, or components that are common to both sexes but are expressed
12 in different amounts (Kent et al., 1984; Celerier et al., 1993; de Oliveira et al., 1999; Binford,
13 2001a, Escoubas et al., 2002; Herzig et al., 2008; Herzig & Hodgson, 2009).

14 Sexual dimorphism of traits is generally explained by one of two influences: (1) the
15 ecology or behavior of the sexes differs and the dimorphism optimizes the effectiveness of the
16 traits in both the male and female contexts; (2) the traits are involved in courtship, mating or
17 production of offspring, and males and females differ in reproductive interests (e.g. Darwin,
18 1871; Shine, 1989; Hedrick & Temeles, 1989). Attempts to explain factors underlying sexual
19 dimorphism in venoms generally focus on sexual differences in feeding ecology or predation
20 risk. Standardized comparisons of female venoms of different spider species indicate that
21 differences in chemical composition and/or insecticidal potency tend to correlate with feeding
22 biology (Friedel & Nentwig 1989; Nentwig et al., 1992; Binford, 2001b). Sexual dimorphism in
23 feeding biology (e.g. prey capture behavior, trophic morphology, rates of prey consumption) is
24 well documented across spiders (Walker & Rypstra, 2001; Walker & Rypstra, 2002; Pekar et al.,
25 2011). Therefore, it is reasonable to predict, and generally assumed, that sexual dimorphism in

venom chemical composition reflects differences in feeding biology (e.g. Escoubas et al., 2006).

However, we are not aware of empirical tests of this hypothesis.

Here, we examine the link between venom chemistry and feeding biology using a phylogenetic approach. Our goal is to understand how venom chemistry is modified in pairs of taxa in which the males shift feeding niche upon maturity, and those in which they do not shift niche. This allows us to determine whether changes in adult niche differences between sexes are evolutionarily associated with changes in the degree of sexual dimorphism in venoms.

The spiders

Species from a lineage of Hawaiian *Tetragnatha* provide an ideal framework for investigating sexual differences in venom as a function of differences in feeding behavior. Most *Tetragnatha* species spin orb-webs. As adults, males do not spin webs and instead wander in search of females. A speciose monophyletic lineage of *Tetragnatha* in the Hawaiian Islands, the "spiny-leg clade", has abandoned web-building at all life stages and both sexes wander actively in pursuit of prey (Gillespie 1991a; 2002, 2004), thus reducing their sexual dimorphism in ecology and behavior. A lineage of orb-weaving *Tetragnatha* that is either sister, or very closely related, to these wanderers has also diversified on the Hawaiian Islands (Gillespie & Croom 1995; Blackledge & Gillespie 2004). A third Hawaiian tetragnathid species, *Doryonychus raptor* (Simon, 1900), which is thought to be the closest relative of *Tetragnatha* (Gillespie et al. 1997), has also abandoned web-use, and both males and females impale prey from the air with elongate tarsal claws (Gillespie, 1991b, 1992a). Previous comparisons of female venoms of orb-weaving and wandering *Tetragnatha* have shown that venoms of wanderers have a reduced concentration of low molecular weight components (<14 kDa) relative to orb-weavers (Binford 2001b).

In the present study, differences in venom between the sexes was compared in species representing orb-weaving and wandering lineages, and in non-Tetragnathid relatives across a spectrum of phylogenetic divergence and degree of sexual disparity in feeding ecology. The

goals of these comparisons were: (1) to characterize differences in venom composition between sexes within each of these species; and (2) to determine whether the evolutionary decrease in intersexual differences in prey capture behavior in Hawaiian *Tetragnatha* was coincident with an evolutionary decrease in venom dimorphism.

MATERIALS AND METHODS

Species sampled

All spiders used in this study were collected in the field as adults. Voucher specimens are deposited in the Essig Museum, University of California, Berkeley.

Hawaiian Tetragnatha

Spiders were collected in the Hawaiian Islands during June and July, 1994, and May through July, 1996. Males and females of the following species were collected from windward east Maui on the Nature Conservancy of Hawaii's Waikamoi Preserve: the wandering species *T. quasimodo*, *T. waikamoi*, and *T. brevignatha* (Gillespie, 1991a) were collected near Carruther's Camp along the boardwalk at 1876 meters; the orb-weaving species *T. stelarobusta* (Gillespie, 1992b) were collected in lower Waikamoi (1340 m) along Maile Trail. Another orb-weaving species, the undescribed morphospecies referred to as "emerald ovoid" was collected from the summit of Mt. Ka'ala Natural Area Reserve (1220 m) in the Waianae mountain range on the island of Oahu.

Outgroups

The degree of sexual dimorphism in venoms of Hawaiian *Tetragnatha* was compared to that of four other species. (1) A mainland orb-weaving *Tetragnatha*, *T. versicolor* (Walckenaer), was collected from Molino Basin, Santa Catalina Mountains, Arizona, November, 1996. (2) The Hawaiian *Doryonychus raptor* (Tetragnathidae) (Simon, 1900), which does not build a web, was

collected from Hono O Na Pali Natural Area Reserve on Kauai; July, 1996. (3) Another Hawaiian endemic, the large, sheet-web building, in the genus *Orsonwelles* (Linyphiidae) (Hormiga, 2002) was collected from Pahole Natural Area Reserve on Oahu; July, 1996, and (4) the funnel-weaving spider *Eratigena agrestis* (Agelenidae) (Walckenaer, 1802; Exline, 1951; Bolzern et. al. 2013) was collected from an abandoned shipyard in Marysville, Snohomish County, Washington; August, 1998.

Venom Collection and Analysis

Venom collection

Field collected spiders were anesthetized with CO₂, and venom was extracted by means of electrical stimulation as in Binford (2001b). Venom was stored in 0.1% acetic acid (for HPLC separation Mike Adams, pers comm) and frozen at -80° C until analyses which were performed within two years of sample collection. Samples were pooled among individuals matched by species and sex. Pooling was necessary to accumulate enough venom protein to standardize protein separations across species. Pooled samples, while erasing variation among individuals within populations, represent population-level composites that are appropriate for investigating differences between sexes and species. Venoms from multiple independent groups of individuals were analyzed for each sex and species. The number of individual venom samples per pooled collection varied because of differences in the availability of spiders, death of individuals within groups, and occasional failure of a spider to yield venom.

Spiders were milked once before being fed in the lab. They were fed a variety of insects (mostly dipterans and lepidopterans) one day after each milking, and were milked 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 it was milked.

Comparative analyses of venoms

The concentration of proteins in each sample was estimated by measuring absorbance at 280 nm using a Pharmacia Genequant spectrophotometer. To convert this to protein concentration, absorbance was divided by 0.8, the absorbance of bovine serum albumen.

SDS gel electrophoresis

Venom components were separated by one of two types of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation methods. The first used an 8 to 20 % polyacrylamide gradient (Hames & Richwood 1981). This separated proteins larger than 14 kDa, but peptides below that size were not well separated. Because a high concentration of the venom consisted of components <14 kDa, most samples were separated using tricine-sodium dodecyl sulfate TSDS-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 35 µg of crude venom. Gels were stained with silver stain, included 10 lanes with low range standards (2.75 to 43 kDa) (Gibco) on the far left lane and high range standards (14.3 to 200 kDa) (Gibco) on the far right lane, and were run using a tall, mini-gel apparatus (9 x 5 x 0.1 cm) (Hoeffer).

Gel quantification

Sizes and concentrations of components were analyzed for each venom separation. Sizes were estimated by comparing migration distances of bands relative to the migration distances of standards of known size. Concentrations of bands were estimated by integrating the intensity of the bands using BioImage Whole Band Analysis software. From these data, the percent integrated intensity was estimated for each band. Differences between sexes were compared based on the apparent presence and absence of bands of the same size, and on differences in relative amounts of regions of band. To quantify differences between sexes, the average total percent intensity of groups of polypeptides of different size regions were compared using MANOVA analyses on arcsine transformed data with sex and feeding mode as effects. To

control for differences between males and females in the number of samples separated by electrophoresis only pairs of male and female samples from the same species were used for analyses. Comparisons of the presence and absence of bands between species within sexes was difficult in many cases because small samples of venoms for some species did not enable multiple gel separations that were necessary to be confident in the consistency of detection of components present in low concentrations.

The degree of sexual differences for a given species was estimated by subtracting the male percent intensity of different size groups of components from female percent intensity of the same components within each species. These were compared between Hawaiian *Tetragnatha* and outgroup species using Student's t-tests on the average for both of these groups.

To interpret variability, matrices of the percent intensity of regions of bands were analyzed using principal component analysis.

Behavioral and Ecological Analysis

Taxonomic composition of prey captured

Samples of natural prey were collected directly from the chelicerae of adult male and female spiders that were found feeding in the field. Prey remains were taken to the laboratory and identified to the finest possible taxonomic resolution. Live specimens of the most numerous prey types were collected and preserved as voucher specimens.

RESULTS

Venom composition comparison between sexes and species

Venom protein yield

Comparisons of the average amount of protein collected per milked individual indicate that both sex and feeding mode had a significant effect on the absolute yield of venom ($\mu\text{g}/\text{milking}$) (ANOVA, male 12.4, female 8.2, $F_{1,10} = 4.68$, $p = 0.05$; orb 14.9, wanderer 5.7, $F_{1,10} = 22.5$, $p = 0.001$). However, neither sex nor feeding mode was a significant factor when these values were standardized by an estimate of spider size ($\mu\text{g}/\text{milking} \cdot (\text{spider length (mm)}^3)^{-1}$). (male 0.077, female 0.024, $F_{1,10} = 1.06$, $p = 0.33$; feeding mode, orb 0.076 wanderer 0.025, $F_{1,10} = 0.97$, $p = 0.35$). There were no significant differences in whole venom protein yield between sexes of wandering species ($\mu\text{g}/\text{milking}$, $F_{1,4} = 0.48$, $p = 0.51$; $\mu\text{g}/\text{milking} \cdot (\text{spider length (mm)}^3)^{-1}$ $F_{1,4} = 0.99$, $p = 0.35$). Absolute amounts of protein per milking were higher in male than in female orb-weavers ($\mu\text{g}/\text{milking}$, $F_{1,4} = 9.14$, $p = 0.03$), but this difference did not persist when size was considered ($\mu\text{g}/\text{milking} \cdot (\text{spider length (mm)}^3)^{-1}$ $F_{1,4} = 1.08$, $p = 0.41$).

Electrophoretic analyses

Representative electrophoretic separations of all venoms analyzed are shown in a phylogenetic context in Figure 1. A representative SDS-PAGE gel is shown in Figure 2 to illustrate accurate molecular weight comparisons within a gel. At least 26 distinct bands were detected in crude venoms from females, and 38 from males. In both sexes these ranged in size from 3 kDa to 230 kDa. Some sexual dimorphism in venoms is apparent in all species. The degree of the dimorphism differed across species with *Tetragnatha* species (excluding *T. brevignatha*) being strikingly dimorphic and non-*Tetragnatha* species being less dimorphic (Figure 1). Within *Tetragnatha*, males of all of the orb-weaving species analyzed, and males of the wandering species *T. quasimodo* and *T. waikamoi*, have many high molecular weight protein bands (at least 8 between 150 to 230 kDa). Four of these (160, 165, 175 and 190 kDa) were unique to male venoms. Four high molecular weight protein bands, (150, 200, 210 and 230 kDa) were detected in venoms of a few orb-weaving females in very small quantities relative to the males (Figures 1 and 2, Table 1). No proteins larger than 150 kDa were detected in venoms of

wandering females. No proteins larger than 120 kDa were detected in either sex of *T. brevignatha* (n=3), *D. raptor*, *Orsonwelles* sp., or *Tegenaria agrestis*.

Comparisons of the average percent intensity of different size regions of components in male and female Hawaiian *Tetragnatha* are summarized in Table 1. In all *Tetragnatha* species and sexes, the highest concentrations of components were between 3 kDa and 14 kDa. When the rest of the venom profile was considered, regions of highest concentration in females in decreasing order were between 35 - 80 kDa, 14 - 35 kDa, >80 kDa. In contrast, the highest concentrations of components in male venom (except *T. brevignatha*) in decreasing order were >80 kDa, 14-35 kDa and 35-80 kDa. Peptides < 14 kDa were present in greater abundance in female than in male venoms (Table 1). Combined averages of all male samples were significantly lower in concentration of components < 14 kDa than the average for combined orb-weaver females (t-test, $p < 0.001$, 39 df), as well as the average for combined wandering females (t-test, $p = 0.018$, 35 df). Male venoms had significantly more proteins > 80 kDa than did female venoms (Table 1). None of these differences were significant when data for *T. brevignatha* were included.

The average differences between sexes in concentration of venom components of different molecular weights are summarized in Table 2 for *Tetragnatha* species and non-*Tetragnatha* species. The sexual differences in concentrations of components > 80 kDa were significantly larger for *Tetragnatha* than for non-*Tetragnatha* when *T. brevignatha* was excluded from the analysis. However, no regions differed significantly when *T. brevignatha* was included in the analyses.

Principal components analyses of average percent intensity of different size regions of proteins (Figure 3) did not cluster venoms of males and females of *Tetragnatha* species together, except for *T. brevignatha*. Male and female venoms of non-*Tetragnatha* species were more similar to each other than were those of *Tetragnatha* species, however, sex differences among

Orsonwelles sp were nearly as large as those for *T. stelarobusta* along the second principle component. Replicate samples of female and male *T. quasimodo* clustered near one another providing a standard of replicability of this analysis.

Taxonomic composition of prey

The percentage of different prey types found in the chelicerae of male and female spiders in the field is summarized in Table 3. This includes all Hawaiian *Tetragnatha* species for which data are available for both males and females. In all species, there was large overlap in the types of prey consumed by males and females. Moreover, the relative amounts of particular prey types were similar between the sexes in all cases for which there are reasonable sample sizes. In all species, fewer males than females were found with prey.

DISCUSSION

This study reports striking intersexual differences in venom chemical composition in all species of the genus *Tetragnatha* analyzed except *T. brevignatha*. Venoms of *Tetragnatha* sexes are more dimorphic than those of non-*Tetragnatha* species included in this analysis. The dimorphisms are the result of both a smaller concentration in males of low molecular weight components (peptides), and the presence of high molecular weight components in males that are absent, or present in very low concentrations, in females (Figures 1 and 2, Table 1). The expression of abundant high molecular weight components in male venoms but not in females is unlike intersexual differences previously reported in spiders (Kent et al., 1984; Celerier et al., 1993; de Oliveira et al., 1999; Binford, 2001a; Escoubas et al., 2006; Herzig et al., 2008; Herzig & Hodgson, 2009). Based on comparative evolutionary inferences detailed below, we conclude that differences in feeding biology are not the most likely explanations for the high molecular components in male venoms and discuss other possibilities including a role in mating biology.

High molecular weight proteins in male venoms appear to have originated within the *Tetragnatha* lineage. Given our taxon inclusion, the appearance of these proteins is coincident with the origin of adult niche dimorphism of orb-weaving females and wandering adult males. However, our results did not support our hypothesis that intersexual differences in venoms reflect optimization of venoms for feeding niche dimorphism. Rather, a consistent pattern of sexual dimorphism persists in the wandering "spiny-leg clade" of *Tetragnatha* in which both males and females capture prey by wandering and eat the same types of prey (Table 3). The only intersexual difference we detect in feeding biology is that in all species fewer males were found with prey (Table 3). We cannot make strong conclusions about male diet because sample sizes are small, and spiders and prey were not systematically collected to account for background abundance. However, male spiders commonly consume fewer prey than females (eg Walker & Rypstra, 2001) or do not feed at all (Foelix, 2010), so relatively low numbers of males found with prey may reflect real differences in feeding rates between sexes.

The lower concentration of peptides in male relative to female venoms parallels differences among female venoms of Hawaiian *Tetragnatha* species with contrasting feeding ecologies where wandering species have lower concentrations of peptide components than orb-weaving species and consume a wider range of prey types (Binford, 2001b). Spider venom peptides < 14 kDa are often neurotoxins that immobilize prey (Kuhn-Nentwig et al., 2011; Smith et al., 2013). The sexual dimorphism in peptides may reflect selection that optimizes venoms for fine-scale dietary niche differences of males versus females through modifications in expression of specific neurotoxins, or expression levels of shared neurotoxins. While our sample sizes of prey for males are limited (Table 3), the most abundant prey types are shared between sexes for most species, and for taxa with reasonable sampling (> two prey) the types of prey eaten by males closely overlap those of females. So, while more data are needed, if feeding ecology is influencing the reduction in peptides in males, it is more likely to reflect differences in feeding rates than diet.

1 A different possible adaptive function of male specific proteins related to adult niche
 2 dimorphism is that they are used in defense against predators and sexes differ in predation risk.
 3 This role has been proposed to explain higher venom potency against vertebrates in male *Atrax*
 4 species relative to females (Atkinson, 1981; Atkinson & Walker, 1985). For *Tetragnatha*, an
 5 anti-predator function is plausible given different adult niches of male and female orb-weavers,
 6 however it is unlikely for wandering *Tetragnatha* since males and females should be exposed to
 7 the same predation risks. Hawaiian *Tetragnatha* are strictly nocturnal, presumably in response to
 8 avian predation pressure (Perkins, 1913). Spiders of both sexes remain motionless between dawn
 9 and dusk on stems or leaves that match their background (Gillespie, 1991a, Carter 2009).

10 While the dimorphism of high molecular weight proteins in most *Tetragnatha* venoms is
 11 difficult to explain based on differences in adult niche, it could reflect differences in feeding
 12 biology caused by morphological dimorphism in chelicerae and fangs. All *Tetragnatha* have
 13 enlarged, sexually dimorphic adult chelicerae in which males have longer fangs (Levi, 1981).
 14 Fangs of *Tetragnatha* females fit within the cheliceral groove (row of teeth where prey are
 15 crushed) whereas fangs of males are curved and do not fit within the cheliceral groove.
 16 *Tetragnatha* crush prey with their chelicerae (Binford, 2001b) and thus male fangs may be less
 17 effective in prey immobilization than female fangs. If high molecular weight components are
 18 insecticidally active, they could compensate for ineffective crushing in males. However, it is
 19 unlikely that selection would favor unique insecticidally toxic components in males if they have
 20 decreased foraging demands relative to females. A comparison of venom between penultimate
 21 males and females whose chelicerae are monomorphic, and adult males and females, would
 22 clarify whether high molecular weight venom components are unique to sexually mature males.

23 It is possible that male specific high molecular weight venom components do not confer a
 24 unique adaptive function in males. The dimorphism could reflect differences in protein
 25 processing in which large proteins in male venoms are unprocessed, non-functional
 26 preproteins that are cleaved and functional in female venoms. Proteolytic cleavage products

could contribute to both the higher concentration of components <14 kDa in females relative to males, and the lack of the same high molecular weight components in female venom. However, both the uniformity of pattern across species and the high concentrations of male specific components are inconsistent with this argument. Moreover, the cost of producing proteins that serve no functional role in males would favor any selection, however weak, to reduce or eliminate expression. The presence of the dimorphism in *T. versicolor* indicates that the origin of the male-specific components predates the establishment of the radiation in the Hawaiian Islands, estimated at > 5mya (Casquet et al. 2015), and has been subsequently retained, which is unlikely for a nonfunctional protein.

We consider a possible functional role of venom components in mating biology to be a plausible non-feeding related explanation for the unique proteins in male *Tetragnatha* venom. Male and female *Tetragnatha* engage in “cheliceral-locking” during mating in which the dimorphic chelicerae are intertwined with the tip of the male fang placed in or near the cheliceral groove of the female, meaning venom components could readily be exchanged during mating. While similar intrasexual cheliceral interaction could allow the male specific components to play a role in male-male competition, such interactions have never been observed in *Tetragnatha* (West and Toft, 1999). So, could the male-specific venom components serve to subdue the female, or even for mate recognition? Such a role for venom components is rare for any venomous animal and currently unknown in any spider; however, there are several examples in scorpions (Polis and Sissom, 1990), making such a function plausible.

The lack of striking dimorphism in *Doryonychus raptor* becomes particularly interesting when we consider a sexual recognition role to explain the high molecular weight components in venoms of most male *Tetragnatha* we examined. *D. raptor* is a species closely related to *Tetragnatha* that also mates by locking chelicerae. The presence of the dimorphism in *T. versicolor* from Arizona indicates that either the origin of cheliceral mating predates the origin of the dimorphism (Figure 1), or the dimorphism is broadly present in closely related tetragnathid

genera and has been lost in *D. raptor*. Either way, if our conjecture is that sexual dimorphism in venom is associated with cheliceral locking, how might we explain the lack of dimorphism in *D. raptor*? Interestingly, this species is one of very few spiders in which males engage in cheliceral locking with other males (Gillespie 1991c). The behavior is much like male/ female coupling with neither participant showing aggression during the interaction, and movement of the palps resembles that of a male-female encounter suggesting the interaction is simply mistaken identity. The potential lack of recognition of sexual identity in *D. raptor* is at least consistent with the sexual dimorphism in *Tetragnatha* venoms having evolved as a mechanism to facilitate recognition. Several other genera in the family Tetragnathidae, *Pachygnatha*, *Dolichognatha*, and some *Leucauge* also entwine their chelicerae while mating (Álvarez-Padilla 2009), and may represent at least two independent origins of this behavior (but see Eberhard and Huber 1998). Cheliceral locking in *Leucauge marina* facilitates mechanical stimulation of females by setae on male chelicerae during mating, which increases male fitness (Aisenberg et al., 2015), demonstrating the potential for cheliceral contact to influence sexual dynamics in these taxa. It would be informative to investigate the degree of dimorphism of venoms in other cheliceral-locking tetragnathid taxa.

T. brevignatha also lacks striking dimorphism of high molecular weight components in male venoms. As with *D. raptor*, this is intriguing but in a different way: In *T. brevignatha* a heterochronic shift has been implicated in the evolution of the color as well as the much shorter chelicerae in this particular species, compared to close relatives (Carter 2009, Brewer et al. 2015). It would be highly illuminating to determine whether the decrease in sexual differences in the venom could be another outcome of the inferred heterochronic shift.

Conclusions

In summary, this study identifies a sexual dimorphism in venoms of *Tetragnatha* species that is not easily explained by sexual niche differences. We are pursuing proteomic and

transcriptomic analyses to detail the chemical nature of this dimorphism. Combined with the comparative evolutionary framework detailed here, we will be well positioned to infer the functional role of sexually dimorphic components, and better understand the ultimate causes underlying intersexual venom differences. The patterns we observed suggest that venom components may function outside of prey capture or defense against predators, and have particular potential for roles in intraspecific interactions including sexual biology. Within tetragnathid spiders the cheliceral locking behavior during mating provides a physical mechanism for directly transferring venom to the female and facilitates the potential for components secreted in male venoms to contribute to sexual stimulation, nuptial gifts and/or mate recognition. As such, venoms may represent character suites that evolve under combined influences of natural selection for feeding biology and sexual selection. If true, venom phenotypes have exciting potential to be involved in the adaptive radiation of lineages such as Hawaiian *Tetragnatha*.

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Table 1. Average and standard error of concentrations (percent intensity) of different size regions of proteins from female and male *Tetragnatha* excluding *T. brevignatha*. No comparisons were significant when *T. brevignatha* was included. MANOVA analyses were conducted on arcsine transformed data, and only on 3 of 4 size range categories in a given analysis (a constraint of using percentages). Analyses were repeated sequentially excluding each category. The p-value for % 14-35 is from a separate analysis than the one that yielded the p-values for the other 3 categories. When %>80 kDa or <14 kDa data were excluded the whole model was not significant. Results of a whole model MANOVA analysis that excluded data for %14-35 kDa are listed below.

size range (kDa)	females	males	sex effect test	
			F _(1, 6)	p=
% <14	72.8(3.7)	52.3 (7.1)	8.81	0.025
% 14-35	9.34 (2.3)	12.2 (2.3)	0.06	0.824*
% 35-80	13.7 (1.6)	10.5 (1.8)	1.39	0.308
% >80	3.9 (1.3)	25.0 (6.9)	17.35	0.007

MANOVA		
	F	p
whole model	5.51 _{6, 6}	0.028
sex	21.21 _{2, 3}	0.017
feeding	4.26 _{2, 3}	0.133
sex x feeding	0.57 _{2, 3}	0.615

Table 2. Average differences between sexes (females – males) within species of *Tetragnatha* and non-*Tetragnatha* in concentration of venom components analyzed with and without inclusion of *T. brevignatha*. P-values for degree of difference comparisons between *Tetragnatha* and outgroups were from Wilcoxon tests. The comparisons significant at $p < 0.05$ after Dunn-Sidák correction are in bold.

size range (kDa)	<i>Tetragnatha</i> (f-m)	non- <i>Tetragnatha</i> (f-m)	p-values without <i>T. brevignatha</i>	p-values with <i>T. brevignatha</i>
% <14	20.6 (5.8)	5.30 (2.4)	0.052	0.233
% 14-35	-2.86 (2.7)	-3.37 (3.1)	0.859	1.00
% 35-80	3.24 (1.4)	-0.07 (0.5)	0.112	0.25
% >80	-21.9 (6.2)	0.03 (0.05)	<0.001	0.23

1   **FIGURE CAPTIONS**

2   Figure 1. *A composite of phylogenetic relationships among the species* included in the analysis
3   based on Gillespie et al. (1997), Gillespie & Roderick (pers comm), and Scharff &
4   Coddington (1997). Male and female electrophoretic profiles are included for each
5   species. Molecular weights are not standardized across gel images within this figure.
6   Branch shading indicates feeding biology: black = orb-weaving, white = wandering,
7   obliquely striped = sit-and-wait impalers, grey = sheet-web, zig-zag = funnel-webs.

8   Figure 2. *A representative gel analysis* contrasting venom between sexes within species. Note
9   that less than the standard amount of *T. brevignatha* female venom was loaded on this
10   gel.

11   Figure 3. A plot of the *first and second principal components* from analyses of the average
12   percent intensity of size regions of protein bands. Open symbols indicate male samples,
13   filled or shaded symbols indicate female samples.

14

Figure 1

Figure 2

Figure 3

1 **Table 3.** Percentages of different prey types collected from the chelicerae of male (m) and female (f) Hawaiian *Tetragnatha*. Sample
2 sizes are in parentheses. *Data from females are from Binford, 2001b. Prey types shaded in grey are non-flying.

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SPIDERS	PREY											
	Lepidoptera	FLYING		Homoptera	Psocoptera	Psyllidae	CURSORIAL					
		Tipulidae	Diptera Other Diptera				Lep larvae	Diptera larvae	Coleoptera larvae	Araneae Other spiders	Tetragnatha	
<u>ORB-WEAVERS</u>												
<i>T. stelarobusta</i> f (31)*	71	10	6	3	6	3						
<i>T. stelarobusta</i> m (2)	100											
<i>T. eurychasma</i> f (18)*		50	17	17				5				
<i>T. eurychasma</i> m (9)	11	55	11	11				11				
<u>WANDERERS</u>												
<i>T. quasimodo</i> f (47)*	15		15	11	2	2	28	2	8	8	8	
<i>T. quasimodo</i> m (18)	17		28	5		11	27	5	5			
<i>T. kamakou</i> f (23)*	4	9	17	12				26	4	9	4	4
<i>T. kamakou</i> m (6)	33				33				33			
<i>T. waikamoi</i> f (17)*	6	12	18	23	12	6	12				6	6
<i>T. waikamoi</i> m (14)		43	14	7	14	7	14					