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An analysis of geographic and intersexual chemical variation in venoms of the spider *Tegenaria agrestis* (Agelenidae)

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Abstract

The spider *Tegenaria agrestis* is native to Europe, where it is considered medically innocuous. This species recently colonized the US where it has been accused of bites that result in necrotic lesions and systemic effects in humans. One possible explanation of this pattern is the US spiders have unique venom characteristics. This study compares whole venoms from US and European populations to look for unique US characteristics, and to increase our understanding of venom variability within species. This study compared venoms from *T. agrestis* males and females from Marysville, Washington (US), Tungstead Quarry, England (UK) and Le Landeron, Switzerland, by means of liquid chromatography; and the US and UK populations by insect bioassays. Chromatographic profiles were different between sexes, but similar within sexes between US and UK populations. Venoms from the Swiss population differed subtly in composition from UK and US venoms. No peaks were unique to the US population. Intersexual differences were primarily in relative abundance of components. Insect assays revealed no differences between US and UK venom potency, but female venoms were more potent than male. These results are difficult to reconcile with claims of necrotic effects that are unique to venoms of US *Tegenaria*.

Keywords: Spider; Venom; Variation; Population; Sex; Comparative

1. Introduction

Spider venoms are very diverse in chemical composition and modes of action (McCrone, 1969; Geren and Odell, 1984; Atkinson and Wright, 1992; Adams and Olivera, 1994; Shultz, 1997). Moreover, it is becoming increasingly evident that variability in spider venoms is present among recently diverged taxa as well as within species (McCrone and Netzloff, 1965; Russell and Buess, 1970; Atkinson, 1981; Atkinson and Walker, 1985; Muller et al., 1989; Barbaro et al., 1996; Escoubas et al., 1997; de Andrade et al., 1999; de Oliveira et al., 1999; Escoubas et al., 1999). Differences in chemical composition among venoms that confer differences in physiological effects on a given target animal can be due to the presence or absence of particular components, or to shared components that differ in relative abundance. Variability in venoms between congeneric species and between populations and individuals of the same species has not been well studied, yet understanding variability at this level can serve at least three useful functions. First, it can lead to the discovery of rapidly evolving toxins with different neurophysiological effects. Second, it can lend insight into the evolutionary processes and patterns that have given rise to higher level diversity in toxins. Third, it is essential for understanding risk, appropriate treatment and antivenom use when species produce toxins that are medically significant (Chippaux et al., 1991).

The spider species *Tegenaria agrestis* (Walckenaer, 1802) is particularly interesting for investigations of venom variation within species. *T. agrestis* individuals are native to Europe and western central Asia where they are common and widespread. Individuals typically build funnel webs in a range of open, often disturbed habitats (Hänggi et al., 1995). This species was introduced into the Pacific Northwest region of the US in the early 1900s and was first formally identified in the 1930s (Exline, 1936, 1951). The species' range has since expanded to British Columbia, Alaska, Oregon, Idaho, Montana and Utah

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(West et al., 1984; Baird and Akre, 1993; Roe, 1993). By the 1960s, individuals were commonly collected in and around human habitations. Since the early 1980s medically significant bites (causing severe necrosis and systemic effects) have been attributed to *T. agrestis* (Vest, 1987a; Akre and Myhre, 1991; Vest, 1996). This species is also a recent introduction into the UK, where it was first reported in 1949, and is often found in close association with people (Merrett, 1979). Throughout Europe there have never been reports of medical problems associated with bites from these spiders (Bücherl, 1971; Bettini and Brignoli, 1978; Cloudsley-Thompson, 1993). Furthermore, there is evidence that bites of males from the Pacific Northwest have more severe necrotoxic effects on mammalian tissues than do bites from females (Vest, 1987b). Thus, there are hints of geographic and intersexual variability in the effects of *T. agrestis* venom on mammalian tissues.

There are at least four possible explanations of the emergence of medical significance in US *T. agrestis*: (1) an evolutionary change in the venom composition of the ancestors of the US population may have resulted in the origin of a novel necrotoxic effect on mammals; (2) venom chemistry might not differ between US and European spiders, but the populations might differ in habitat or in behavior such that it is more likely that US *T. agrestis* will encounter humans and bite them (Akre and Myhre, 1991); (3) perhaps there are no differences in venom chemical composition or in ecological circumstance and the necrosis is caused by something extrinsic to, but uniquely associated with, the venom of spiders in the US (i.e. a bacterium); and (4) *T. agrestis* do not directly or indirectly cause the necrotic lesions and have been falsely accused.

This study compares the chemical composition of whole venom of male and female *Tegenaria agrestis* from US and European populations. My goals are twofold: (1) to gain a general understanding of variation in venom chemical composition within the species; and (2) to determine whether there are unique components, or differential expression of components in US *T. agrestis* venom that might explain the novel appearance of this species as a medical concern in the Pacific Northwest of the US.

2. Methods

2.1. Spider collection

All spiders were field collected by visually searching under rocks, wood and debris. Ontogenetic and seasonal variation in spider venom potency is known in some spiders (Atkinson and Walker, 1985; Malli et al., 1993; de Andrade et al., 1999). Care was taken to minimize such sources of variation by: (1) collecting within small geographic areas (single populations); (2) using only individuals collected as adults except where noted (males identified by enlarged, sclerotized pedipalps; females by sclerotized epigyna); and (3) restricting the collecting period to August through mid-September, the mating season for this species. European *T. agrestis* were collected in August 1997, from two sites in England, and one site in Switzerland. Most of the analyses were conducted using venom of spiders from a regenerating quarry site at Tungstead Quarry, 3 km east of Buxton in central England. A small number of spiders were also collected from an abandoned woodlot in central Liverpool. In western Switzerland spiders were collected along a roadside 1.5 km north of Le Landeron. The Swiss spiders and some female Tungstead Quarry spiders were collected as penultimate adults and molted to adulthood in captivity. Venom from these individuals was analyzed separately from venom from individuals collected as adults. US spiders were collected from an abandoned shipyard in Marysville, Snohomish County, Washington, in September 1997, and August 1998. For outgroup comparisons (intended to provide a comparison for interpreting the extent of differences within *T. agrestis*) *Tegenaria gigantea* (Chamberlin and Ivie, 1937) were collected at the same site as *T. agrestis* in Marysville.

While collecting in the field, general descriptions of habitat and web locations, and any evidence of prey in the chelicerae or in the web were recorded.

Spiders were housed in individual deli cups (8.5 cm diameter × 7 cm tall, 250 cm^3 volume) and kept at 25°C under a 12:12 light:dark cycle at the University of Arizona, Tucson, AZ.

2.2. Venom collection

Spiders were anesthetized with CO_2, and venom was extracted by means of 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 the spider's mouths. The yield of venom per spider was measured as the venom was drawn into a calibrated microcapillary tube. 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. To obtain sufficient quantities of venom for analyses, samples were pooled from individuals matched by population and sex (average 9.1 ± 2.5 individuals/pooled sample). The number of individual venom samples per pooled collection varied slightly because of attrition within groups (death of individuals) 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. When there were sufficient numbers of individuals, multiple groups of individuals were pooled within each population and sex. This provided comparisons of poolings among different groups of individuals from within the same population, and among sequential collections from the same groups of individuals over time.

Field-caught spiders were milked once before being fed in the lab. Spiders were then fed crickets 24 h after milking and

were not milked again until a minimum of 7 days after feeding. Crude venom samples were stored at −80°C until analysis.

2.3. Chemical analyses

Venom components were separated by reverse-phase high performance liquid chromatography (RP-HPLC) (Hewlett-Packard HP1050 with a diode array detector). The use of RP-HPLC maximized resolution and repeatability. A 3-μl aliquot of crude venom was used for each separation. Samples were centrifuged and filtered through 0.2 μm syringe filters (Alltech). Samples were chromatographed on an Applied Biosystems Aquapore Brownlee RP-300 C8 column, (220 × 4.6 mm), with a guard column, using acetonitrile with 0.085% trifluoroacetic acid (TFA) as the mobile phase, and constant 0.1% TFA and water. The mobile phase was ramped from 3 to 53% acetonitrile across 67 min (0.75% min^{-1}) then increased to 80% across 5 min and held at 80% for 15 min [Fig. 1(c)]. To try to improve resolution in the most congested regions of the chromatogram (between 20 and 40 min), the ramping gradient was changed to 3–15% acetonitrile across 20 min, 15–30% across 30 min, 30–37.5% across 10 min, extended to 100% in 5 min and held for 10 min [Fig. 2(d)]. Absorbance was measured at 220 and 280 nm. Fractions were collected and stored for bioassays and further purification of selected components. A known amount of bovine serum albumin (BSA) was run on the same column using the same elution profile at the beginning of each day of analysis.

The total amount of venom components in each sample was estimated by integrating the area under HPLC chromatogram curves of absorbance at 220 nm. Peptides, proteins and polyamines absorb at 220 nm, making this a measure of total component amount, whereas 280 nm is more selective for proteins and peptides. Total chromatogram areas were translated into total amounts of components using a regression of absorbance peak areas (220 nm) of known amounts of BSA analyzed using the same HPLC gradient protocol as that used to separate the venom samples (peak area = 531 (μg protein) + 1280; $n = 16$; $r^2 = 0.36$). The estimated values are similar to measures of protein concentration of US and UK *T. agrestis* samples obtained from a BCA method (Pierce) (H. Gomez, pers. comm.) and from absorbance at 280 nm (pers. obs.). This method of quantifying component concentration was used to conserve venom from populations for which it was limited.

2.4. Statistical analyses

The chemical compositions of the venom pools were compared by visually aligning chromatogram peaks. Retention time, relative peak height, and peak shape and area were criteria for alignment. The author assumed aligned peaks across samples contained the same components. More detailed analyses (mass spectrometry or sequence analysis) are necessary to verify this assumption. Resolution differed slightly on different days of HPLC analysis. This generally was due to peak widening, with large peaks differentially masking the visibility of adjacent smaller peaks. To control for this variability, pairs of chromatograms contrasting populations and sexes that were analyzed on the same day. To avoid pseudoreplication, only HPLC chromatogram data for one venom pooling from a given group of spiders were included.

To interpret variability visually, matrices of aligned peak percent areas were analyzed using principal component analysis. These analyses included either all peaks, or peaks representing >1% of the total peak area. The latter analysis included all peaks that were consistently visible, whereas including all peaks incorporated variability in visibility of minor peaks that may have be a function of minor variations in resolution. Significant differences in the average amount of the same peak as a function of population, sex, collection year, or maturity status when collected were determined using pairwise Student's *t*-tests and the Dunn–Sidák correction for multiple comparisons. Differences between samples as a function of milking sequence (field caught, first, second, or third milking) were analyzed using ANOVA. Differences between populations and sexes in venom yield and concentration were analyzed using ANOVA and pairwise Student's *t*-tests. All analyses made use of the statistical program JMP (SAS).

2.5. Venom functional assays

Relative potency and insecticidal bioactivity were assayed by injecting crude venom into larvae of the housefly *Phaenicia sericata* (average weight of 30 randomly selected individuals was 21.6 ± 6.9 mg). Crude venom was diluted in 0.1 N ammonium acetate pH8.5 to concentrations of 50, 25, 12 and 6%. A 0.2 μl aliquot of each dilution of venoms from male and female spiders of the Marysville and Tungstead Quarry populations, as well as an ammonium acetate control were injected into a minimum of eight larvae. Samples were injected into the pronotum using a 10 μl Hamilton syringe with a 33 gauge needle and a 0.2 μl increment syringe dispenser. Injected larvae were observed continuously for the first 5 min after injection, and then activity was noted every 5 min for 30 min, after 1, 1.5, 2, 24 and 48 h. Doses at which 50% of the larvae were paralyzed (PD_{50}) were estimated using probit analysis (SAS).

Venom effects were also noted during observations of captures of crickets in the laboratory.

3. Results

3.1. Spider natural history and collecting notes

T. agrestis were abundant at the Marysville and Tungstead Quarry sites. Adults webs were under large rocks, pieces of wood, and miscellaneous paper, plastic, and rubber debris. Juveniles were found on webs in more exposed areas

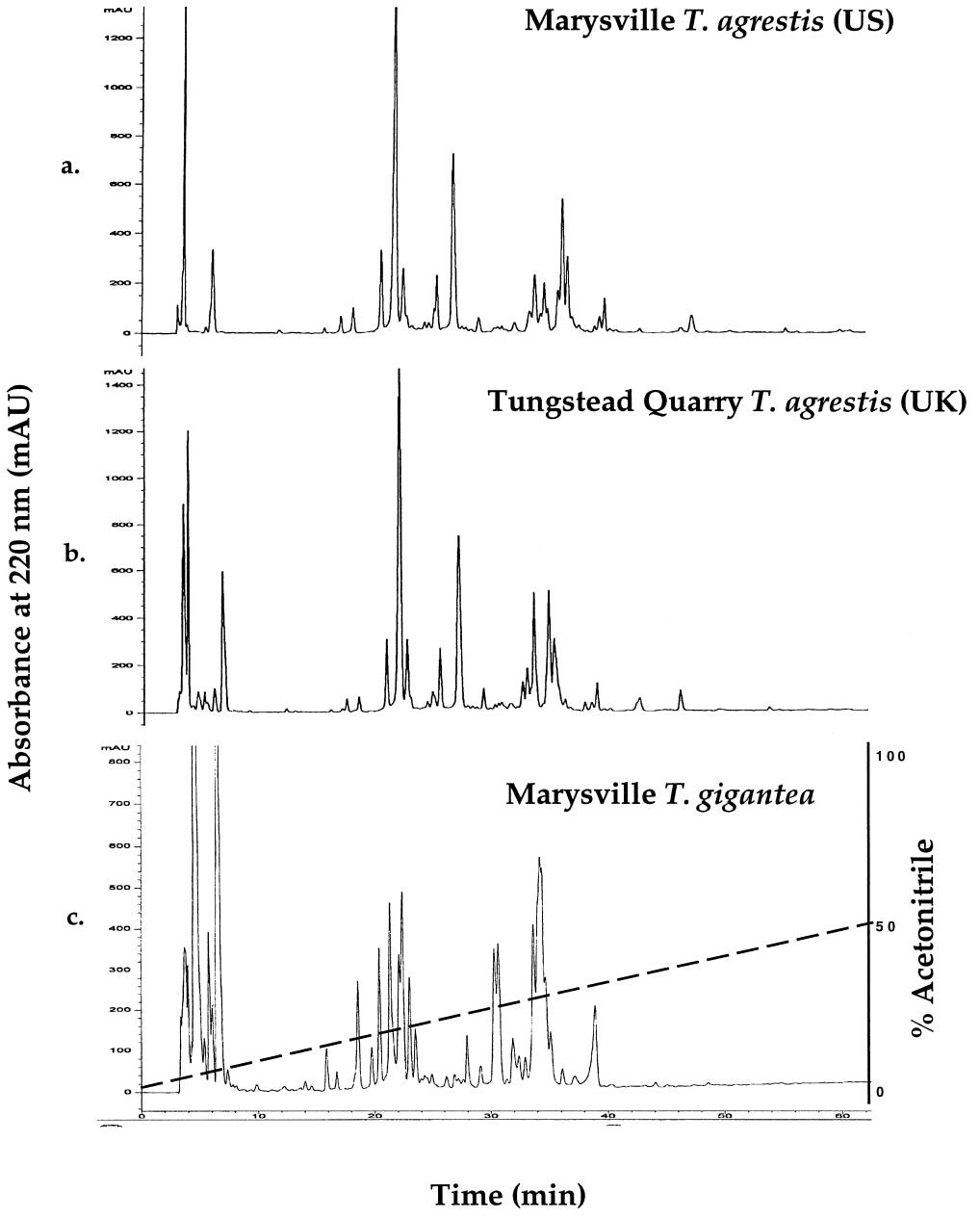

Fig. 1. Aligned chromatograms from RP-HPLC analyses of venom from: (a) female *Tegenaria agrestis* from Marysville, Washington (US); (b) female *T. agrestis* from Tungstead Quarry, England; and (c) female *T. gigantea* from Marysville, WA.

including clumps of grasses and in abandoned rabbit holes (Tungstead Quarry). *T. agrestis* at the Swiss site were less abundant and found beneath rocks along a rural roadside. Natural prey remains found in the webs or in spider's chelicerae included two black ants (*Lasius niger*), one red ant (*Myrmica* sp.), one 5 mm elytron of a beetle, an asilid fly, a bibionid fly and a large tipulid from Tungstead Quarry; and one ant (*Camponotus* sp.), two tenebrionid beetles, and a syrphid fly from Marysville. Although these data are few, they indicate *T. agrestis* at both sites are, at least, generalist insect predators.

3.2. Venom yield and concentration

Venom volumes obtained from spiders and venom total concentration estimates are listed in Table 1. Both population and sex significantly effected *T. agrestis* venom yield (ANOVA, Table 1). Standardization by spider body length

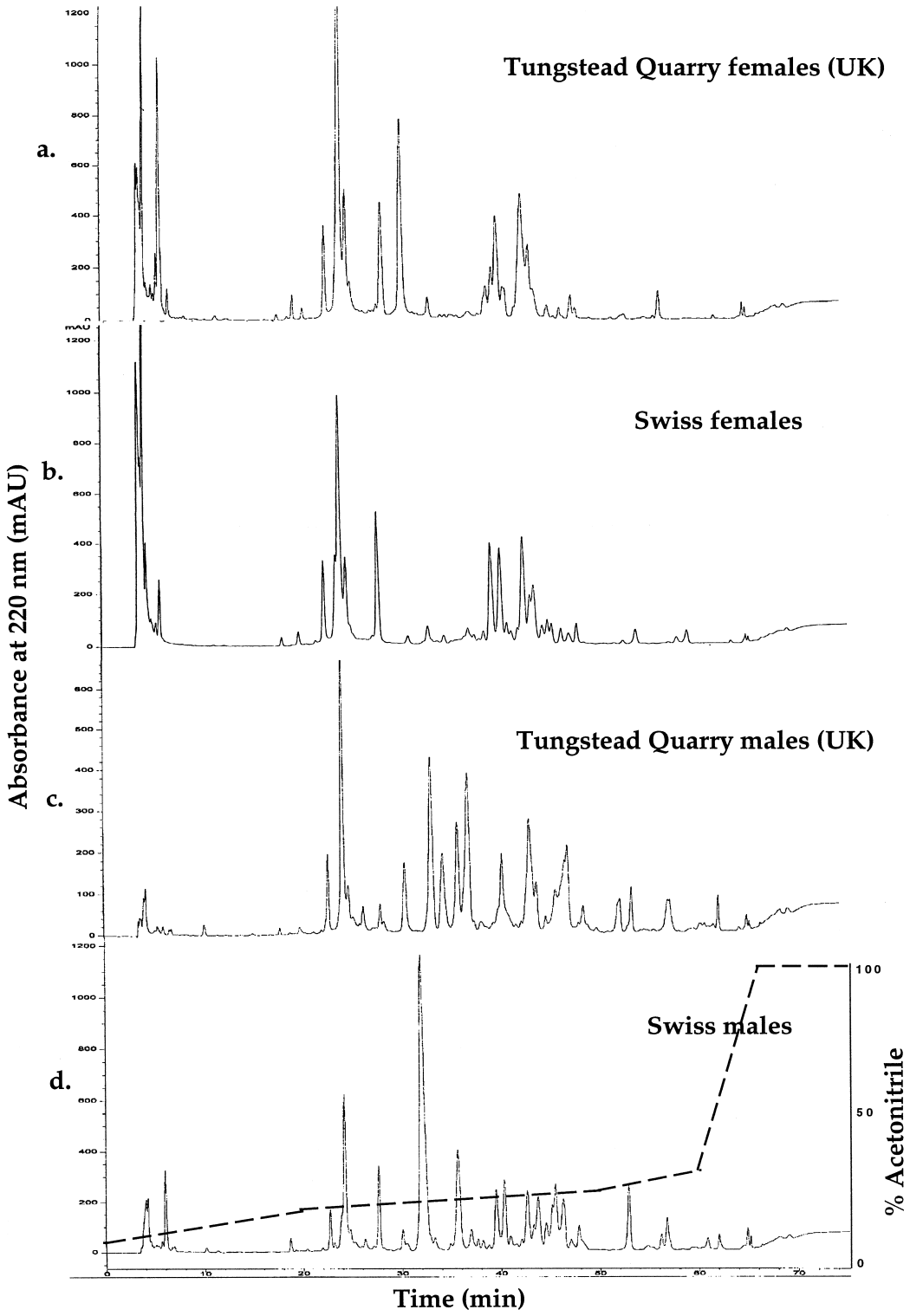

Fig. 2. Aligned chromatograms from RP-HPLC analyses using the extended acetonitrile gradient (d.) for *T. agrestis*: (a) Tungstead Quarry females; (b) Swiss females; (c) Tungstead Quarry males; and (d) Swiss males.

Table 1
Venom protein concentration (μg/μl) and per spider venom yield (absolute μl, and standardized by total spider length in mm) for female (f) and male (m) US, UK, and Swiss *T. agrestis*, and US *T. gigantea*. Numbers are means with standard error in parentheses and sample sizes below. Sample sizes differ because concentration estimates are based on pooled samples and yield is based on total numbers of spiders milked. Results are listed below from ANOVA analysis of the effects of population and sex on absolute volume of venom per spider for the *T. agrestis* populations

Venom	US *T. agrestis*		UK *T. agrestis*		Swiss *T. agrestis*		*T. gigantea*	
	♀	♂	♀	♂	♀	♂	♀	♂
Concentration (μg/μl)	100.9 (8.1)	95.6 (19.5)	106.1 (8.9)	118.5 (5.4)	98.6 (26.7)	141.6	119.4 (34.9)	109.6
	13	5	11	4	2	1	2	1
Yield per spider (μl)	0.75 (0.03)	0.26 (0.05)	0.80 (0.05)	0.43 (0.04)	0.27 (0.04)	0.11 (0.02)	0.91 (0.09)	0.69 (0.15)
	154	19	64	41	18	16	27	10
Yield per spider standardized (μl/mm spider length)	0.063 (0.002)	0.028 (0.005)	0.076 (0.004)	0.046 (0.003)	0.033 (0.005)	0.012 (0.002)	0.062 (0.006)	0.056 (0.010)
	154	19	64	41	18	16	27	10
Source	df	Sum of squares	F ratio	$P > F$				
pop	2	0.467	2.27	0.0398				
sex	1	8.107	74.20	< 0.001				

and spider body length cubed [a proportional estimate of mass (Thompson, 1942)], and exclusion of the Swiss population from the analysis had no effect on the significance of the population or sex effect ($P < 0.0001$ in all cases). Females produced more venom than males in all *T. agrestis* populations (*t*-tests: Marysville $P < 0.001$; Tungstead Quarry, $P < 0.001$; Swiss, $P < 0.002$). Absolute yields of venom from *T. gigantea* were also higher for females than for males (*t*-test; $P = 0.05$, but this result did not persist when standardized by spider body length (*t*-test; $P = 0.845$) or body length cubed ($P = 0.220$) (Table 1).

There were no significant differences in venom total component concentrations between Marysville, and Tungstead Quarry *T. agrestis* populations (*t*-test, females, $P = 0.67$; males, $P = 0.34$) or between sexes (*t*-test, US $P = 0.76$; UK $P = 0.43$). The number of estimates of venom protein concentration for Swiss populations and *T. gigantea* were too small to include in statistical analyses.

3.3. Venom HPLC comparisons

Typical chromatograms from Marysville, Tungstead Quarry and Swiss *T. agrestis* females and males, and from female *T. gigantea,* are shown in Figs. 1 and 2. Relative elution times of given peaks appeared to be the same between the original and the extended gradient. In total, 83 peaks were detectable in *T. agrestis* venoms. For discussion, peaks that represented >1% of the total area (38 peaks, accounted for 89.4 ± 3.6% of the total area) were arbitrarily labeled based on elution time (Fig. 4). Subtle variability in resolution (peak widening) across chromatographic runs made visibility of some peaks inconsistent. Therefore only a subset of the total number of peaks was used in the statistical analysis (see Section 2).

3.4. T. gigantea vs T. agrestis

T. agrestis and *T. gigantea* venoms differed in composition, both in relative amounts of apparently shared components and in the likely presence of unique components. Peaks that were either unique to, or more highly expressed in, *T. gigantea* eluted primarily between 15 and 25 min (Fig. 1). The consistently dominant peak (peak number 8) in *T. agrestis* male and female venoms also eluted in this region. Chromatograms from *T. gigantea* were not aligned peak-by-peak with *T. agrestis* because the degree of difference made such alignments dubious without identification of specific components through mass spectrometry or sequence information (Fig. 1). Therefore, differences between these species were not quantified.

3.5. Variability within T. agrestis

Venom profiles were consistently similar within and between the Marysville and Tungstead Quarry populations of *T. agrestis* for a given sex [Figs. 1(a), (b) and Fig. 3(a)], but the Swiss population differed from the other populations

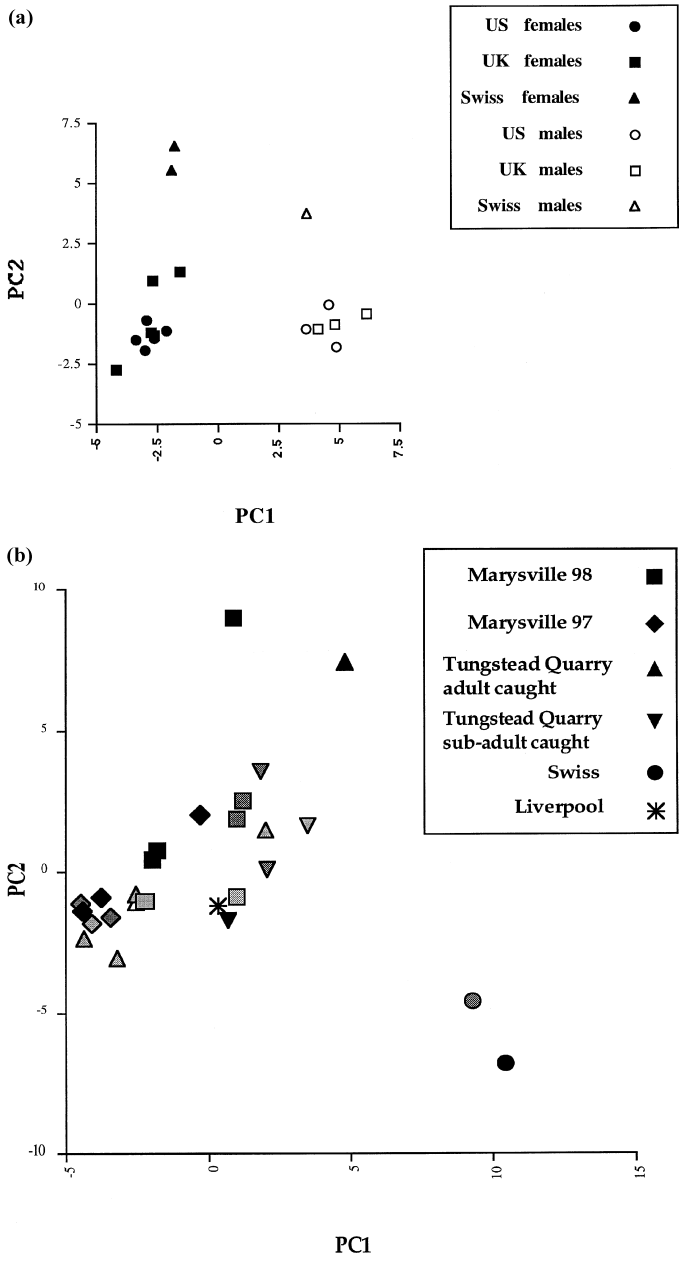

Fig. 3. First and second principal component scores from analyses of HPLC peak% area of venoms. (a) Includes venoms of males and females from Marysville, English and Swiss populations. Only peaks that represent >1% of the total area of the chromatogram for any population by sex group average were used in the analysis (41 out of 82 peaks). This represents 89.4 ± 3.6% of the total area. (b) Includes all peaks from all female samples from the Marysville, English and Swiss populations. Symbols are shaded black for the first milking and get lighter for consecutive milkings. Marysville samples are separated by year of spider collections, and Tungstead Quarry samples are separated by animals that were collected as adults in the field and animals that were collected as subadults and molted to adulthood in the lab.

in both sexes [Figs. 2 and 3(a)]. The Swiss population was distinguished primarily by the second principal component (PC2) [Fig. 3(a)], that accounted for 15.5% of the variability. Sexes cluster distinctly and were separated primarily by the first principal component (PC1) [Fig. 3(a)] that accounted for 33.3% of the variability in the data set. Ninety-five percent of the variability in the data was explained only after 12 principal components. The Swiss population was distinguishable from the other two populations by differences in relative amounts of shared

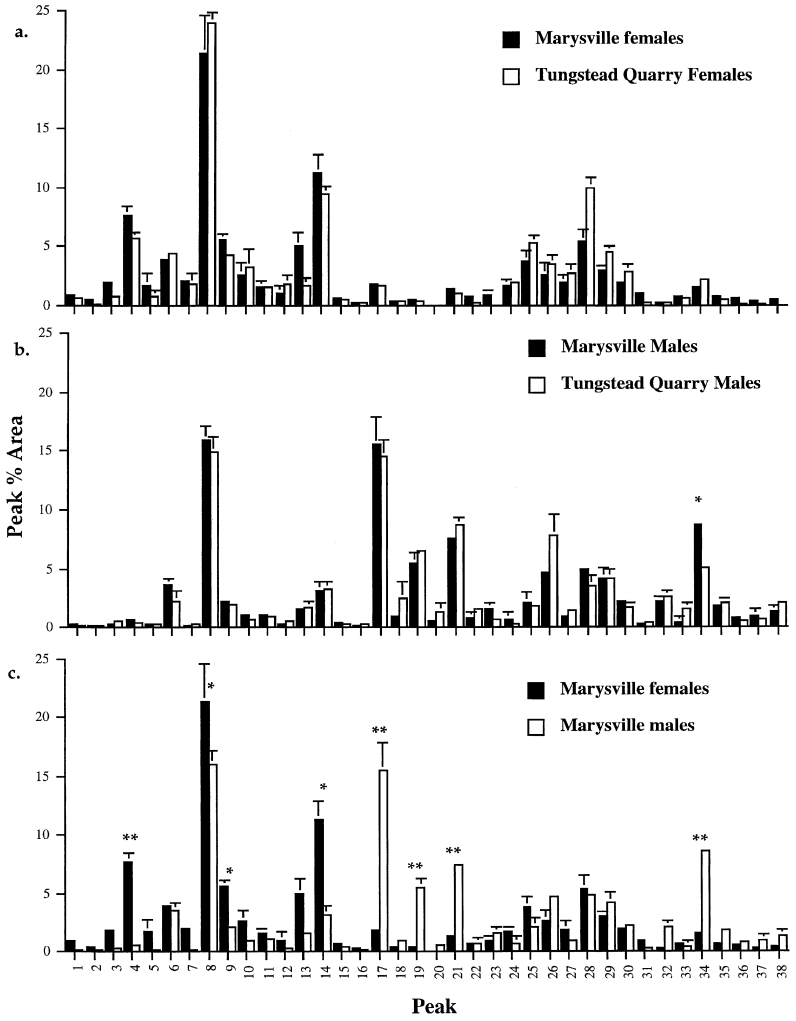

Fig. 4. Comparisons of population by sex average% areas of individual HPLC peaks representing >1% of total peak area (error bars represent standard error). a. compares *T. agrestis* females from Marysville ($n = 5$) and Tungstead Quarry ($n = 5$); (b) compares *T. agrestis* males from Marysville ($n = 3$) male and Tungstead Quarry ($n = 3$); (c) compares Marysville *T. agrestis* females ($n = 5$) and males ($n = 3$). Differences in peak area were analyzed using student *t*-tests with a Dunn–Sidák correction for multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$.

components, and by three components that appeared to be unique to the Swiss population. One unique component (S1, Table 2) was a relatively large peak making up 4.43% of the Swiss female venoms and 0.46% of the Swiss male venoms. The distinction between the Swiss and the other populations should be interpreted with caution because the result is based on venom pools from one sample group each of males and females of this population (one milking pool from males, and two separate milkings from the same group of females).

Peak-by-peak comparisons identified no peaks that were unique to the Marysville population. Two minor peaks (<1% total area) were inconsistently identified as unique to the Tungstead Quarry females in analyses using the extended gradient (Table 2). Comparisons of individual peak relative abundance identified one peak present in significantly higher concentration in Marysville males than in Tungstead Quarry males [peak 34, Fig. 4(b)]. No peaks differed significantly in abundance between females from Marysville and Tungstead Quarry [Fig. 4(a)].

Differences between sexes were primarily in relative amounts of peaks that were present in both sexes [Figs. 2 and 4(c)]. Eight peaks differed significantly between the sexes in relative amounts [Fig. 4(c)]. Four of these were more abundant in male than in female venoms [Figs. 2 and 4(c), peaks 18, 20, 22, and 35]. Four peaks were more abundant in female than in male venoms [Figs. 2 and 4(c), peaks 4, 9, 10 and 15]. There were four minor peaks (<1% total area) unique to females and one minor peak unique to males (Table 2). Given homology of the peaks was not

Table 2
Average percent of total chromatogram area of all aligned RP-HPLC peaks that are unique to any population or sex. Peaks found in England (UK) and Swiss (S) populations, but not the US are labelled 'E'

	♀1	♀2	♀3	♀4	♂1	S1	S♀1	S♀2	E♀	S + ♂	♂ + E	♀ + UK♂
Females												
Seattle	0.28	0.22	0.41	0.06								0.48
UK	0.35	0.23	0.66	0.47					0.20		0.35	0.27
Swiss	0.47	0.46				4.43	0.54	1.29	0.65	0.41	0.77	0.92
Males												
Seattle					0.97					0.41	0.71	
UK					0.95					0.42	0.58	0.77
Swiss					0.96	0.46					0.71	

certain, this should be verified with mass spectral or sequence information.

Principal components analysis including all female samples yielded no obvious distinctions between female venoms as a function of collection year (97 or 98, Marysville females), sequential milkings in the lab (Marysville and Tungstead Quarry females), and maturity status when collected in the field (adults vs penultimate instar that molted to adulthood in the lab, Tungstead Quarry females) [Fig. 3(b)]. The only female sample from field-caught adult Liverpool spiders was not distinct from the Marysville and Tungstead Quarry samples [Fig. 3(b)]. The first and second principal components in this analysis explained 19.75 and 13.76% of the total variation respectively. Ninety-five percent of the total variation was explained only after the first 18 principal components.

Peak-by-peak comparisons identified no significant differences in relative abundance of particular peaks as a function of collection year, maturity status or milking sequence.

3.6. Venom function

Casual observation during lab feeding indicates that the sequence of events during captures of cricket prey by *T. agrestis* consists of spiders running out of retreats toward prey once they are detected in their silk webs, biting the prey, and holding them in their jaws. Silk was not used during the initial capture, nor did spiders macerate prey, indicating that venom is the primary tool for subduing prey. Only when prey were paralyzed did the spiders release and tether them with silk before transporting them into their funnel retreat for consumption. This sequence of events was the same for male and female *T. agrestis* from all three populations. Bites typically targeted the thorax. Prey were generally paralyzed within the first 30 s of being bitten, but in one instance a bitten cricket prey struggled for over 1 h. The prey capture behavioral sequence was the same for *T. gigantea.*.

The paralytic effect of venom delivered both by natural bites and by injection were the same in terms of sequence of paralysis. Prey were paralyzed instantly and irreversibly in prey removal assays and by injections at high concentration. Hearts of envenomated prey continued to beat for >48 h after injection, indicating that the cumulative effect of the venom toxins is not directly lethal. There were no differences in the paralytic effect between Marysville and Tungstead Quarry venoms, or between male and female venoms. To paralyze prey, however, more male venom was required than the more insecticidally potent female venom (Fig. 5). The timing and the sequence of prey paralysis and death were the same for *T. gigantea* ($n = 5$ prey removals).

4. Discussion

This study has revealed remarkable similarity in the composition of venom of *T. agrestis* collected from a recently geographically isolated population in the US and from populations in England. The data also reveal differences between *T. agrestis* and its congener *T. gigantea,* differences within populations between sexes, and slight differences between the Marysville and Tungstead Quarry

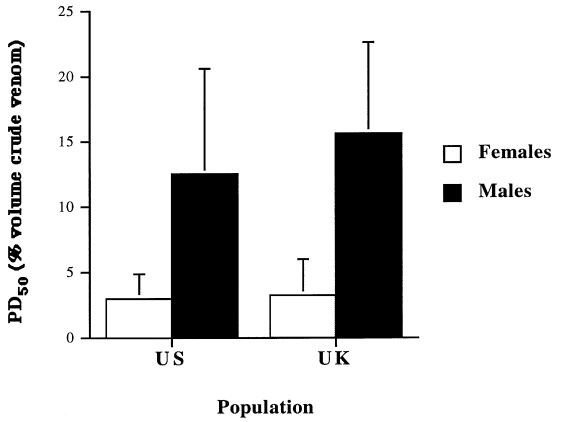

Fig. 5. The doses (% volume crude venom) at which 50% of injected *Phaenicia sericata* larvae were paralyzed by venom from Marysville and Tungstead Quarry, male and female *T. agrestis.* Error bars represent one standard deviation from the mean.

populations and the Swiss population. Both the variability, and the lack thereof, have implications which will be addressed in turn.

4.1. Interspecific differences

Venom composition of *T. agrestis* shows both similarities to and differences from that of *T. gigantea* (Fig. 1). For the purposes of this study, the differences serve as benchmarks for illustrating the lack of difference between *T. agrestis* populations. Differences between these species are interesting in themselves for a number of reasons. First, the most striking differences are due to the presence of more early-eluting peaks (<20% CH_3CN) in *T. gigantea* venoms than in *T. agrestis* venoms. Components that elute under these conditions in other spiders are often polyamine-toxins (Adams et al., 1989; Skinner et al., 1989; Quistad et al., 1991; Escoubas et al., 1997) which are rare among animals, yet seem to be relatively common and diverse in spider venoms and are potent, target-specific neurotoxins (Blagbrough et al., 1992; Adams and Olivera, 1994; Shultz, 1997). Secondly, the *T. agrestis* and *T. gigantea* populations used in this study are similar ecologically. Both species are recent colonists of the US from Europe, they are sympatric (were found literally side-by-side) on each continent (Leech and Steiner, 1992), they build similar webs and capture prey using the same behavioral sequence. Bioassays are necessary to determine if the differences in chemical composition reflect any functional differences between the venoms. Differences between congeneric species in venom composition and/or toxic activity have been detected in the genera *Brachypelma* (tarantula) (Escoubas et al., 1997; Escoubas et al., 1999), *Atrax* (Atkinson, 1981), *Latrodectus* (widow spiders) (McCrone and Netzloff, 1965; Muller et al., 1989), *Loxosceles* (Barbaro et al., 1996. None of these studies examined differences as a function of ecological circumstance. More detailed analyses of interspecific differences could help determine rates of evolutionary change in venom chemistry.

4.2. Intersexual differences

There are consistent and striking differences in chemical composition, volume per individual, and insecticidal potency between male and female venoms of *T. agrestis* spiders from the same populations. Differences are due mainly to differential expression of common components and not to components that are unique to either sex [Fig. 4(c)], but there are a few minor peaks unique to each sex (Table 2). Differential expression of components could be due either to a plastic response to different immediate functional requirements of the venoms by the different sexes, or to a relaxation of, or change in the role of venom in one sex or the other. As adults, males seek out females, mate, and then senesce and die within a few months. Females remain on webs, mate, lay and incubate eggs, and overwinter as adults after their first mating season. The direct fitness benefits of capturing prey for yolk deposition and overwintering in females may be greater than the benefits for prey capture in males at this time. Thus one might expect higher expression of insecticidal toxins in females than in males (Malli et al., 1993). Males may be more directly subject to predation pressure than females as they wander in search of mates, and selection could favor expression of toxins targeted to defense against predators that might include vertebrates (Atkinson and Walker, 1985).

Male and female *T. agrestis* venoms are similar in concentration (Table 1), yet female venoms are more insecticidally potent than males (Fig. 5). Sexual differences in insecticidal potency are not necessarily matched by differences in systemic or necrotic toxicity in mammals. Assays conducted by Vest (1987b) suggest that *T. agrestis* male venoms are more toxic to mammals than female venoms. Johnson et al. (1998) identified peptide toxins in *T. agrestis* venoms that are insect specific (masses 5643, 5679, and 5700 Da) and mammalian specific (masses roughly 5000 and 9000 Da, Johnson, pers. comm.). Differential sex-specific expression of these target-specific toxins could explain the functional differences. The mammalian toxins reported by Johnson et al. (1998) are of comparable size to mammalian specific toxins from another agelenid species, *Agelenopsis aperta* (Mintz et al., 1992), but differ from two proteins (66,000 Da) that Vest (1993a) reports to be expressed in higher quantities in male than in female *T. agrestis* venom. Mice assayed by Johnson et al. (1998) died within 1 h (Johnson, pers. comm.), which is too quickly for necrosis to occur. Thus, Johnson et al. (1998) did not attempt to corroborate direct assays demonstrating necrosis (Vest, 1987b).

Intersexual differences in spider venom amounts and potency are known from most species that have been studied. A common trend is for females to produce more venom than males, even when data are normalized for the size of the spider (Atkinson, 1981; Atkinson and Walker, 1985; Malli et al., 1993; Celerier et al., 1993; de Oliveira et al., 1999). The sex with more potent venom differs across species, and is a function of the target animal (Atkinson, 1981; Kent et al., 1984; Atkinson and Walker, 1985; Malli et al., 1993; Celerier et al., 1993; de Oliveira et al., 1999; but see Newlands et al., 1982) and/or the specific neurophysiological target (Rash et al., 2000). More work relating venom variation to ecology could help to decipher the ultimate causes of intersexual venom differences.

4.3. Interpopulational differences

Within sexes venom composition is strikingly similar between Marysville (US) and Tungstead Quarry (UK) populations, but these populations differ from the Swiss population. Insofar as these populations are representative of the larger contiguous regions from which they come, there are two implications. First, there has been little change in

venoms in either the US or the UK populations since they shared a common ancestor. Like US *T. agrestis*, populations in the British Isles are recent colonists, first formally recognized in the middle of this century. The results here imply that both the US and UK populations have been isolated from the Swiss population for a long time and may be derived from the same, or very similar, source population which is far from Switzerland. Sampling more European populations of *T. agrestis* would help to characterize the amount of variation across this region, and perhaps to localize the source of the introductions. Efforts to find populations of *T. agrestis* in coastal Iberia in habitats similar to those where this species occurs in the US and England were unsuccessful.

The data from this study identify no conspicuous features that distinguish Marysville *T.agrestis* venom from venom of the Tungstead Quarry population and, thus might explain why *T. agrestis* are a medical concern in the US, but not in Europe. No peaks are detectable by this method that are unique to the Marysville population.

There is only one component, peak 34 (Fig. 4), worth consideration as potentially conferring a different effect in the US by being expressed in greater proportions in the US than in the UK. This peak has greater relative abundance in Marysville than in Tungstead Quarry males, and is more abundant in venom of US males than US females (Fig. 4). If this component does have a necrotic effect, its greater relative abundance in males is consistent with the observation of more severe necrosis caused by male bites than female bites (Vest, 1987b). Moreover, the fact that it is present in both sexes is consistent with necrosis occurring year round (Vest, 1987a, 1989) even though adult males are only present in late summer and early fall. However, the argument does not explain why necrotic lesion forming bites have not been observed in Europe given that males from Tungstead Quarry produce significantly more of peak 34 than Marysville females. This is true for percent of total venom composition ($P = 0.007$, *t*-test) and for estimates of absolute amounts of peak 34 per HPLC sample (3 μl venom) using peak area regression estimates (see Section 2) (Tungstead Quarry males $= 8.67 \pm 1.39$ μg protein; Marysville females $= 3.37 \pm 0.67$ μg protein; $P = 0.002$, *t*-test). Furthermore, because females produce more venom than males, the amount of peak 34 injected into bite victims could be similar between the sexes. Therefore, this component is not effectively expressed in higher amounts in the US than in the UK. Given this lack of difference, and that no other components have larger quantities in the US, some other difference must explain the lack of necrotizing bites in Europe.

The lack of evidence of components unique to, or more highly expressed in the venom of US *T. agrestis* individuals narrows the possible explanations for the suspected recent association of *T. agrestis* with necrotic arachnidism in the US but not in the species' native European range. If a necrotizing agent is intrinsic to the venom, that same component is present at least in the UK, and perhaps throughout Europe, but has not been the source of any clinical issues. Either European and US *T. agrestis* differ in habitat in a way that makes it less likely that European *T. agrestis* will encounter and bite people, or *T. agrestis* bites do occur in Europe and result in necrosis, without the link having been noted. Given the frequency of alleged *T. agrestis* bites in the US (Vest, 1987a; Akre and Myhre, 1991; Vest, 1996), the relative scarcity of unexplained necrotic arachnidism in Europe (see Maretic and Russell, 1979 for a single case) indicates that, if bites occur from this species, they are more frequent in the US. The possibility that European and US populations differ in habitat in a way that makes this more likely will be addressed in another paper.

An alternative possibility is that there is no necrotoxin intrinsic to *T. agrestis* venom, but the necrotizing agent is produced by a bacterium that is uniquely associated with the US population (on fangs or chelicerae). The possibility that necrotic arachnidism could be caused by infection of the bite site with the bacterium *Mycobacterium ulcerans,* known to cause slow-developing ulcers on human skin, was proposed by Harvey and Raven (1991). Atkinson et al. (1995) provide several lines of evidence that a consistent direct association between *M. ulcerans* and spiders is biologically unrealistic. Moreover, antibacterial peptides have recently been discovered in spider venoms (Yan and Adams, 1998; Haeberli et al., 2000). These findings make it unlikely that microbial activity is responsible for necrosis resulting from spider bites.

A final possible explanation is that *T. agrestis* are not directly or indirectly the cause of necrosis and have been falsely accused. Misdiagnosis of spider bites is a well known and common issue (Russell and Gertsch, 1983; Kunkel, 1985; Rosenstein and Kramer, 1987; Kemp, 1990; Vest, 1993b; Vetter and Visscher, 1998). Agents that create symptoms that are often confused with spider bites are discussed in the above references, and range from reactions to other organisms (arthropods, such as the lyme disease vectors (*Ixodes* spp.); fungal dermatophytes; viruses; bacterial infections), to systemic reactions to underlying medical problems (leukemia, infections). Nonetheless, Vest (1993b) advises: "The tentative diagnosis of necrotic arachnidism should be considered in any clinical case in which deep, slow-healing necrotic lesions develop, with or without systemic manifestations, unless clear evidence of another cause can be demonstrated". There are a number of reasons why Vest's advice to diagnose necrotic arachnidism as a default is not reasonable. First, there are no definitive diagnostic characteristics that are absolutely and uniquely associated with the bite of a given spider, making accurate diagnosis of spider bites dependent on retrieval and identification of spiders that were seen biting the victim (Russell and Gertsch, 1983). The author is aware of only one case in the literature where a *T. agrestis* individual was directly linked to a bite which allegedly led to a necrotic lesion (Vest, 1987a; Akre and Myhre, 1991; Fisher et al.,

1994; Vest, 1996). Even this case is doubtful because the victim did not seek medical assistance (and presumably spider identification) for 79 days after the bite (Case 2, Vest, 1996).

A second reason to hesitate before a diagnosis of necrotic arachnidism is that biting humans is outside of normal biological activity for spiders except in defense. Defensive bites risk spider's lives and tend to occur only when the spider is at risk of being crushed. *T. agrestis* has been described anecdotally as unusually aggressive (Akre and Myhre, 1991). However, after handling over 500 adult *T. agrestis* the author has seen no displays of aggression (threat postures, attempted bites) in these spiders.

Corroboration of solid, direct evidence of the involvement of *T. agrestis* bites in creating necrotic lesions and systemic effects, and solid empirical evidence that bites are likely to be common, are both essential before it can be justified that any cases of necrosis in the Pacific Northwest are diagnosed by default as necrotic arachnidism caused by *T. agrestis*. Rapid diagnosis of necrotic arachnidism without direct, solid evidence and a full diagnostic work-up can lead to inappropriate treatment and the lack of consideration of more severe underlying medical issues (Kunkel, 1985; Rosenstein and Kramer, 1987; Kemp, 1990; Koh, 1998).

In summary, this study finds evidence of variability in venom composition between geographically isolated populations and between sexes of *Tegenaria agrestis*. The geographic variability, however, is not as expected between US and UK populations, but between these populations and the only population investigated on mainland Europe. There is no evidence of rapid evolutionary divergence of the recently isolated US population from the UK population. This calls into question the presumption that there is a difference in the medical effects of US and UK *T. agrestis* bites and illuminates a need for further mammalian assays of both US and UK venoms.

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