

Changes in composition of spider orb web sticky droplets with starvation and web removal, and synthesis of sticky droplet compounds

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Accepted 6 January 2006

Summary

The sticky spiral of araneoid spider orb webs consists of silk fibers coated with adhesive droplets. The droplets contain a variety of low-molecular-mass compounds (LMM). Within a species, a fairly consistent ratio of LMM is often observed, but substantial variability can exist. To gain insight into factors influencing LMM composition, spiders of three araneid species were starved and LMM from their webs were analyzed for changes in composition. To determine if these changes were consistent with the spider's ability to synthesize the different organic LMM, synthetic capacities were estimated following the feeding of radiolabeled metabolites. Some changes in droplet composition were broadly consistent with differing synthetic capacities: molar percentages of less readily synthesized compounds (e.g. choline, isethionate, *N*-acetyltaurine) typically declined with starvation, at least during a portion of the imposed fast, while more readily synthesized compounds (e.g. GABamide, glycine) tended to increase. Most striking was the apparent partial substitution of *N*-acetylputrescine by the more readily synthesized GABamide in fasting *Argiope trifasciata*.

However, departures from expected compositional shifts demonstrated that synthetic capacity alone does not adequately predict sticky droplet compositional shifts with starvation. Moreover, feeding controls exhibited some changes in composition similar to starving spiders. As the webs of both feeding and starving spiders were removed for chemical analysis and could not be recycled, the loss of LMM contained in these webs likely contributed to similarities between treatments. In addition, feeding spiders molted, oviposited and/or built heavier webs. The added metabolic demands of these activities may have contributed to changes in composition similar to those resulting from starvation.

Supplementary material available online at
<http://jeb.biologists.org/cgi/content/full/209/8/1463/DC1>

Key words: 4-aminobutyramide, *Araneus cavaticus*, choline, glycine, glycine betaine, isethionic acid, orb web, putrescine, resource allocation, spider web recycling, sticky spiral, taurine.

Introduction

In the construction of its orb web an araneoid spider first builds a non-sticky scaffolding using ampullate silk gland fibers and piriform silk gland junctional cements/attachment disks. One part of the scaffolding, the radii, are arranged like the spokes of a wheel. Subsequently, the sticky spiral (or adhesive or viscid spiral, among other names) (Zschokke, 1999) is attached to the radii. The sticky spiral consists of a pair of flagelliform silk gland fibers coated with an adhesive, aqueous secretion from the aggregate glands (Sekiguchi, 1952; Peters, 1955). This aggregate gland secretion contains organic and inorganic low-molecular-mass compounds (LMM; <200 Da), at least one high-molecular-mass phosphorylated glycoprotein and, likely, lipids (see references in Higgins et al., 2001; Schulz, 2001). Organic LMM include 11 of the 12 compounds monitored in this study; GABamide (4-

aminobutyramide; GAB), *N*-monoacetylputrescine (NAP), isethionic acid (2-hydroxyethane sulfonic acid; Ise), *N*-acetyltaurine (NAT), glycine (Gly), choline (Cho), glycine betaine (Bet), alanine (Ala), proline (Pro), putrescine (Put), taurine (Tau) (Fischer and Brander, 1960; Tillinghast and Christenson, 1984; Vollrath et al., 1990; Townley et al., 1991; Higgins et al., 2001).

The aggregate gland secretion, initially deposited as an unstable liquid cylinder, spontaneously redistributes into a pattern of linked droplets (Boys, 1889; Warburton, 1890; Edmonds and Vollrath, 1992; Ball, 1999). Concurrent with droplet formation is the formation of discrete nodules at the center of the droplets that contain the aforementioned glycoprotein (Vollrath and Tillinghast, 1991). While it is believed the nodules are the principal adhesive agents in the droplets (Richter, 1956; Vollrath and Tillinghast, 1991), the

functions of the LMM have not been established, though one role, adsorbing atmospheric moisture, is indicated by the hygroscopic properties of certain LMM (Fischer and Brander, 1960; Schildknecht et al., 1972; Vollrath et al., 1990; Townley et al., 1991).

The LMM account for about 40–70% of the desiccated mass of orb webs, indicating they are important to web function (Fischer and Brander, 1960; Anderson and Tillinghast, 1980; Tillinghast, 1984; Tillinghast and Christenson, 1984; Townley et al., 1991). They may contribute to the elastomeric mechanical properties of the sticky spiral; directly, by inhibiting crystallization within the flagelliform fibers (Gosline et al., 1995), or indirectly, by adsorbing moisture, thereby keeping the fibers elastomeric through hydration (Vollrath and Edmonds, 1989; Bonthron et al., 1992; Hayashi and Lewis, 1998). Direct and indirect effects of the LMM may also be important to the functioning of the adhesive glycoprotein within the nodules. The conformation adopted by the glycoprotein and the formation of nodules may depend on direct interactions between the glycoprotein and LMM, and the ability of the glycoprotein to spread out on contact with an insect (Richter, 1956; Vollrath and Tillinghast, 1991) presumably relies on the presence of water. Water adsorbed by LMM also promotes droplet formation, which increases adhesiveness (Edmonds and Vollrath, 1992), and contributes to aerodynamic drag following the impact of an insect, thus helping to dissipate the prey's kinetic energy (Lin et al., 1995).

The LMM composition of sticky droplets often differs quantitatively and qualitatively among araneoid species (Vollrath et al., 1990; Townley et al., 1991; Higgins et al., 2001) (M. A. Townley and E. K. Tillinghast, unpublished). The significance of these differences is unknown. One attempt to determine if differences in composition translate into differences in web hygroscopicity among three araneid species did not demonstrate such a relationship (Townley et al., 1991). Nor do we know the extent to which the composition of the droplets is tailored to the physical environment in which a spider forages, the prey captured, or the metabolic needs of the spider. And while LMM composition within a species is consistent enough that analyses of pooled web collections from groups of individuals generally yield similar results (Vollrath et al., 1990; Townley et al., 1991), substantial intraspecific differences have also been observed within and among populations, between the sexes, and following a change in environment/diet (Vollrath et al., 1990; Townley et al., 1991; Higgins et al., 2001).

The goal of the present study was to examine the influence of starvation on the LMM composition of the sticky droplets and to determine if observed changes reflect differences in the spider's capacity to synthesize the various organic LMM. Specifically, we anticipated that the total mass of LMM would decrease in webs of starving spiders, but that there would be greater relative declines in those organic LMM the spider is less able to synthesize. Therefore, in addition to analyzing series of webs built by starving and feeding spiders of three araneid species, we fed radiolabeled compounds to two of

these species to determine to what extent the spiders can synthesize the different organic LMM (Kasting and McGinnis, 1966). Given the results of these synthetic capacity measurements, some of the changes in composition observed with starvation conformed to our expectations, but others did not. Unexpected results also came from the control feeding spiders, which also exhibited changes in droplet composition, in some respects similar to trends seen in webs of starving spiders. Possible explanations for these results are discussed. In addition, the construction of egg sacs by some of the study spiders allowed us to make a preliminary examination of the influence of the egg laying cycle on droplet composition.

Materials and methods

Synthesis of organic LMM by Argiope

Spider collection, maintenance and radioisotope feeding

Adult female *Argiope aurantia* Lucas 1833 and *Argiope trifasciata* (Forskål 1775) were collected in southern New Hampshire, USA from late July to late August and in the latter half of September, respectively. Spiders were housed individually in wood or aluminum frames (51 cm×51 cm×9 cm) with glass plates on the front and back. They were exposed to the prevailing natural light:dark cycle in a room facing east at temperatures and relative humidities that approximated outside conditions.

Four groups of *A. aurantia* (5–7 spiders/group) and three groups of *A. trifasciata* (6–7 spiders/group) were formed. For each *A. aurantia*, 1.1×10^5 Bq D-[U- ^{14}C]glucose (ICN Biomedicals, Irvine, CA, USA) in 3 μl water was placed on the mouthparts using a 10 μl Hamilton syringe (Reno, NV, USA) and the spider was observed until the droplet was completely imbibed. *Argiope trifasciata* were likewise fed D-[U- ^{14}C]glucose, but in quantities of 3.7×10^4 – 1.1×10^5 Bq/spider.

The synthesis of the three sulfonic acids in the webs' sticky droplets (Ise, NAT, Tau) was also investigated by feeding adult female *A. aurantia* (4 groups; 4–6 spiders/group; 3.7×10^4 – 1.5×10^5 Bq/spider) a solution containing L-[^{35}S]methionine and L-[^{35}S]cysteine (TRAN ^{35}S -Label, ICN).

Spiders were either fed radioisotope within 3 days of being captured or, if held longer prior to isotope feeding, were fed crickets or grasshoppers. They were not fed after isotope feeding, but orbs built on the day of isotope feeding were partially collapsed and the spiders were allowed to recycle them. Water was given daily, except for the day before and the day of isotope feeding to encourage drinking of the labeled solution.

Handling of radiolabeled orb webs

The first five webs built by each spider after ingesting radioisotope were collapsed, wound onto one end of a glass micropipet, and stored at -20°C . All webs built by members of the same group were pooled on a single micropipet (19–30 webs/group). Not all spiders built five webs. While still on their micropipets, each group's web collection was extracted twice in 4 ml distilled water for 1.5 h with occasional gentle

vortexing. The two extracts were pooled, dried and analyzed by proton nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$) as described below using the *Argiope* acquisition parameters (see ' $^1\text{H-NMR}$ analysis'). From these analyses molar percentages of the 11 organic LMM (listed in the Introduction) in each of the 11 extracts (seven ^{14}C -labeled, four ^{35}S -labeled) were calculated. Extracts were then fractionated individually by high-voltage paper electrophoresis (HVE).

HVE and chromatography of radiolabeled water-soluble web fractions

Each water-soluble extract was fractionated by HVE at 3000 V on 23 cm \times 57 cm sheets of Whatman 3MM chromatography paper (Brentford, England), with the extract applied in 75 μl of electrolyte solution over a 13 cm long origin. For the ^{14}C -labeled extracts, the origin was 28 cm from the positive pole and electrophoresis was carried out for 35 min. For the ^{35}S -labeled extracts, the origin was 41 cm from the positive pole and the run lasted 55 min. The electrolyte solution used was pyridine:glacial acetic acid:water (133:4.6:1862.4, v/v), pH 6.4. Coolant at 2°C was passed through the unit's lower plate.

After electrophoresis, the paper was air dried and autoradiograms were generated using Kodak BioMax MR film (Rochester, NY, USA). Using the autoradiograms as a guide, the electrophoretograms were cut into radioactive and nonradioactive bands. All bands were eluted overnight with distilled water containing 0.001% sodium azide in a chamber saturated with water vapor. Eluates were dried, resuspended in 1 ml distilled water, and 50 μl removed for scintillation counting. Selected eluates were examined by $^1\text{H-NMR}$ to establish locations of organic LMM.

To determine what percentage of the radioisotope in a HVE eluate was incorporated into an organic LMM of interest, portions of radioactive eluates were further fractionated by two-dimensional thin layer chromatography (2D-TLC) on 20 cm \times 20 cm cellulose plates (0.1 mm thickness, Merck KGaA, Darmstadt, Germany) using pyridine:acetone:ammonium hydroxide:water (45:30:5:20, v/v) in the first dimension and 2-propanol:formic acid:water (75:12.5:12.5, v/v) in the second dimension (Schmidt, 1974). This was necessary because HVE did not resolve all of the organic LMM measured in this study and because these compounds, though they constitute the bulk of the organic LMM, could not be assumed to be the only organic or sulfur-containing compounds extracted by water from orb webs (and, indeed, they are not). Autoradiograms were prepared from the 2D-TLC plates as above. If an autoradiogram revealed two or more radioactive compounds, these were individually scraped off the plate and their radioactivities measured by scintillation counting. Identifications of the organic LMM on the plates were based on NMR of the HVE eluates and on experience in this system with the migration characteristics of most of the organic LMM of interest. Where any doubt remained, radioactive compounds were scraped off plates and examined by NMR to confirm identity.

Using pre- and post-fractionation NMR data and the radioactivity data obtained following HVE and 2D-TLC, relative specific radioactivities for the organic LMM were estimated (initially in c.p.m./mole%, then, for ^{14}C -labeled LMM, converted to c.p.m./molar quantity of carbon).

For the two *N*-acetylated LMM, NAP and NAT, estimates were also made of the relative specific activities of their acetyl groups *versus* their Put or Tau moieties. Because NAP was not detected in webs of *A. aurantia*, only the three *A. trifasciata* water-soluble extracts were used to make this determination for this compound. After identifying the HVE eluates containing NAP and NAT by NMR, 70% of each was hydrolyzed under vacuum (6.7 Pa) in 6 mol l $^{-1}$ HCl at 115°C for 20 h. At the same time, commercial samples of Put and Tau (two each) were treated likewise. Based on the percentage recovery of these standards (Put, 83.8%; Tau, 84.3%), a correction for losses occurring during hydrolysis was made. Hydrolyzed and unhydrolyzed portions of eluates were fractionated by HVE and 2D-TLC and autoradiograms generated. Of the total ^{14}C present in these eluates, the percentage incorporated into the Put/Tau moieties *versus* the intact compounds was determined by scintillation counting of compounds scraped off the 2D-TLC plates. The difference between these was taken to be the percentage of the ^{14}C incorporated into the acetyl groups.

Effects of starvation and web removal on orb web mass and LMM composition in Araneus and Argiope *Collection and maintenance of spiders*

Juvenile *Araneus cavaticus* (Keyserling 1882) were collected from barns in southern New Hampshire, USA between early April and mid-July. Juvenile female *Argiope aurantia* and *Argiope trifasciata* were also collected locally from mid-July to early August and throughout August, respectively. Spiders were housed as described above.

Before spiders molted and were placed in an experimental group, they were fed 1–3 flies on days they built webs, were allowed to recycle (i.e. consume) their webs freely, and were given water daily. Both before and after assigning spiders to groups, *A. cavaticus* and *A. aurantia* were fed house flies (*Musca domestica* L.) and other dipterans [primarily *Phaenicia sericata* (Meigen 1826) and *Phormia regina* (Meigen 1826)], while *A. trifasciata* received house flies exclusively.

Formation of study groups and collection of orb webs

During this study, 21 groups were formed (supplementary material Fig. S1), with the spiders composing a group receiving the same treatment (fed or starved) and being of the same species, sex and stage (juvenile or adult) and, for adult females, having made the same number of egg sacs (0–3). All spiders were added to a group at the same point in the molt/intermolt cycle (beginning of intermolt). With *A. cavaticus*, webs of male and female juveniles (all penultimate instars) and adult females were collected. With the two *Argiope* species, only webs built by adult females were collected. An individual spider could belong to only one group

at a time, but to two or more groups over the course of the study, as explained below.

Orb webs were collected from a spider only once it had reached the desired stage following its most recent ecdysis. From 1–3 molts were required in the laboratory before the desired stage was reached. Spiders were initially divided into a feeding group and a starving group, with the first individual to reach the desired stage randomly assigned to one of these groups. The next individual that molted to the desired stage was then assigned to the other group and this alternation continued as other spiders subsequently molted to the desired stage.

Following ecdysis, with the building of the first orb web, the spider, whether assigned to a feeding or starving group, was fed two or more flies (see above) totaling about 50 mg wet mass. The web was then partially collapsed and the spider was allowed to recycle it. Thus, no compositional or mass data were obtained from the first web. All subsequent webs, however, were collected on micropipets and stored at -20°C . All second webs built by members of the same group were collected on the same micropipet and are referred to as the web 2 collection of the group. Likewise, all third webs built by the spiders within a group were pooled to yield the web 3 collection and so on. The complete set of web collections from a group is referred to as a ‘series’.

Spiders in starving groups were treated differently from spiders in feeding groups after the construction of the second orb web. In both groups, the second webs were collected as described above, but then only feeding group spiders were handed one or more flies, totaling about 25–35 mg wet mass. Feeding group spiders were fed only on days they built a web. All spiders were given water daily. With starving groups, spiders were stressed until resultant changes in behavior/physiology were readily apparent (e.g. sluggishness, construction of incomplete webs), then feeding was resumed, at which point the spiders became members of a ‘resumed feeding’ group (supplementary material Fig. S1).

All constructed orb webs, unless they contained no sticky spiral whatsoever, were collected from each member of a group and added to that group’s series of web collections until one of four events occurred (supplementary material Fig. S1): (1) the spider was transferred to another group, (2) the spider died, (3) the spider escaped, or (4) the date arrived, 30 September, on which other obligations forced us to end web collecting for the year. This lattermost event was not too detrimental as many of the spiders involved, all adult *A. cavaticus* or *A. aurantia*, had already become relatively inactive by this time and would have built few additional webs. However, the series from the *A. aurantia* PES3 group (see next paragraph) was ended prematurely. *Argiope trifasciata* webs were collected during a different year when there was no need to terminate the study on a particular date.

There were three reasons for transferring a spider to another group. Transfer of starving group spiders to a resumed feeding group has already been described. The other two reasons were egg sac construction and molting. Egg sac construction

resulted in a spider being transferred to a post-egg-sac (PES) group. Because some individuals of both *A. aurantia* and *A. trifasciata* produced three egg sacs (containing nonviable eggs), the construction of each of which was followed by the spinning of additional orb webs, there are three successive PES groups for both species (PES1, 2 and 3; supplementary material Fig. S1). Molting pertains to the penultimate instar *A. cavaticus* only since adults do not molt. On reaching adulthood males lack the ability to build orb webs (Sekiguchi, 1955) so the final molt marked the end of their period of service. Female *A. cavaticus*, on the other hand, by the procedure described above, were assigned to either a feeding adult or a starving adult group after their final molt (supplementary material Fig. S1).

Following the transfer of a spider to either a resumed feeding or PES group we did not allow it to recycle its first web, as spiders in starving or feeding groups were allowed, but collected this web and fed the spider one or more flies totaling about 25–35 mg wet mass. In all other ways web collection and feeding were carried out as for members of feeding groups.

Gravimetric measurements of orb webs

Each web collection was scraped off its micropipet with a razor blade, desiccated *in vacuo* over phosphorus pentoxide for at least 2 days, weighed to the nearest 0.01 mg, then extracted three times in 2 ml distilled water for 1 h with occasional gentle vortexing. The three extracts were pooled and dried on a Savant Speed Vac Concentrator (Hicksville, NY, USA), then transferred with two volumes of distilled water totaling 300 μl to a pre-weighed cup fashioned from the cap of a microcentrifuge tube. After desiccating as above for at least 3 days, this water-soluble fraction of the web, containing the LMM, was weighed and then transferred back to its sample tube with three volumes of distilled water totaling 1 ml. After drying, the sample was analyzed by $^1\text{H-NMR}$. The desiccated water-insoluble fraction was also weighed.

Within a group, not all individuals built the same number of webs (supplementary material Table S1). Consequently, later web collections in a series contained fewer webs than earlier web collections. Initially, therefore, anticipating difficulty with $^1\text{H-NMR}$ analysis on the smaller samples (due to unacceptably large numbers of scans being required to achieve a good signal-to-noise ratio), we typically pooled two or more end-of-series web collections to generate water-soluble extracts that would be large enough to allow for analysis in a reasonable amount of time. Note that this more infrequent ‘horizontal pooling’ of, e.g. web collections 16–19, is in addition to the invariable practice of ‘vertical pooling’ of, e.g. all second webs built by the members of a group. We found, however, that our first horizontal poolings were too generous and that smaller samples (0.3–1 mg) could be analyzed within an acceptable amount of time (3 h to overnight). Thus, we did less horizontal pooling as the study progressed. As the first webs analyzed were those built by *Araneus cavaticus*, more horizontal pooling was carried out on webs of this species.

Gravimetric measurements of spiders

Fresh and dry mass data on the three study species were obtained to gain some measure of the percentage of a spider's dry mass that it typically invests in its orb web, thus providing an indication of the loss incurred when, as in this study, a spider is denied the opportunity to recycle its web. Freshly captured local penultimate instar and adult female *Araneus cavaticus*, and adult female *Argiope aurantia* and *Argiope trifasciata* were weighed immediately on return to the laboratory. Some of these individuals were anesthetized with CO₂, immersed in 80% ethanol for 1 h, and dried to constant mass in a 43°C oven. Only adult females that were not conspicuously gravid were collected for these measurements.

Composition of orb web water-soluble fractions

¹H-NMR analysis. The water-soluble fraction of each web collection was dissolved in 0.5–1.0 ml 99.96% D₂O (Cambridge Isotope Laboratories, Andover, MA, USA) and analyzed on a Bruker AM-360 spectrometer (Billerica, MA, USA) with a 5 mm proton selective probe. 360-MHz ¹H spectra were obtained at a temperature of 300K with 2-methyl-2-propanol added as an internal standard ($\delta=1.2200$ p.p.m.). Following Fourier transformation, peak areas were integrated and used to calculate the molar percentages of the 11 organic LMM dealt with in this study. ¹H-NMR chemical shifts and coupling constants for each of these LMM in D₂O have been reported previously (Townley et al., 1991; Higgins et al., 2001).

In all NMR analyses a spectral width of 5000 Hz was examined and a pulse width of 4.3 μ s, yielding about a 53° flip angle, was used. Various numbers of scans (128–5064) were accumulated depending on sample size. Several other acquisition parameters differed between *A. cavaticus* and *Argiope* samples. With the *A. cavaticus* extracts, analyzed first, the pulse repetition time was the same as the acquisition time, 3.28 s, during which 32K data points were acquired and later zero-filled to 64K prior to Fourier transformation. However, analyses of a standard solution containing the 11 organic LMM indicated that a longer time between pulses would yield more accurate molar percentages (supplementary material Table S2), particularly for Pro. Therefore, with the *Argiope* extracts, a longer pulse repetition time was adopted (8.28 s) that still allowed samples to be analyzed within a reasonable amount of instrument time, with 64K data points acquired over 6.55 s, later zero-filled to 128K. We did not attempt to apply corrections to the *A. cavaticus* data. Thus, more than anything else, Pro is likely somewhat underrepresented in the *A. cavaticus* webs, though consistently so.

In addition to measuring relative quantities of the organic LMM in molar percentage, we wanted to estimate absolute quantities of the organic LMM. With the *A. cavaticus* web collections, this was achieved by a standard addition method whereby each sample was analyzed by NMR twice, before and after the addition of synthetic GAB (Kleemann et al., 1980) (1 μ mol mg⁻¹ water-soluble fraction). Integrations from the two analyses allowed us to calculate average μ g web⁻¹ for each

organic LMM in each web collection. We also calculated the percentage of the water-soluble fraction's mass that could be accounted for by the 11 organic LMM. For the two *Argiope* species, we obtained approximate μ g web⁻¹ of the organic LMM by assuming that the percentage of the water-soluble fraction's mass that could be accounted for by the 11 organic LMM was the same as the mean in *A. cavaticus*.

Phosphorus analysis. After NMR analysis, each water-soluble web fraction was assayed for inorganic phosphorus (Chen et al., 1956) using KH₂PO₄ as a standard.

Statistical analyses

Pearson correlation coefficients were calculated from molar percentage data: (1) for all pairwise combinations of individual LMM, with data from all non-radioactive web collections from each species pooled for the analyses, (2) between H₂PO₄⁻ and total positively charged LMM, total negatively charged organic LMM, and 'excess' positive charge (see 'H₂PO₄⁻ and charge balance' in Results), and (3) for all pairwise combinations of web collection number and total readily synthesized, moderately synthesized, and poorly synthesized LMM (see 'Synthesis of organic LMM by *Argiope*' in Results) in web collections from the starving and feeding groups. As in an earlier study (Higgins et al., 2001), all molar percentages were arcsine-transformed prior to analysis and Bonferroni-corrected *P* values are reported.

Linear regression analysis was used to evaluate changes in three quantities over the series of web collections for a group: (1) the percentage of web mass that was solubilized by water, (2) the percentage of the water-soluble fraction's mass that was accounted for by the 11 organic LMM in *A. cavaticus* (see '¹H-NMR analysis' above), and (3) the 'excess' positive charge. A *t*-test was used to determine if slopes of regressions differed significantly from zero. Slopes of 'excess' positive charge regressions were compared using a Tukey's multiple comparison test (Zar, 1999).

Specific radioactivities of LMM from radiolabeled webs were normalized as described in Table 1, log₁₀-transformed, and compared using analysis of variance (ANOVA) and Tukey's HSD multiple comparisons test. The specific radioactivities reported in Table 1 are back-transformed means and 95% confidence intervals.

Transformations and slope comparisons were done in Microsoft Excel 2002 (Redmond, WA, USA); correlations, regressions, ANOVAs and multiple comparisons of means were carried out in Systat 10 (Point Richmond, CA, USA).

Results

Synthesis of organic LMM by *Argiope*

Relative specific radioactivities of the organic LMM from the labeled web collections are presented in Table 1, along with the molar percentages of the organic LMM in these webs. Put and free Pro were not detected by NMR in any of the radiolabeled web collections. Thus, specific activity data were not obtained for these compounds, though the specific activity

Table 1. Molar percentages and relative specific radioactivities of organic LMM in water extracts of radiolabeled orb webs from *Argiope fed [U-¹⁴C]glucose* or a solution containing *L-[³⁵S]methionine* and *L-[³⁵S]cysteine*

	<i>Argiope aurantia</i>			<i>Argiope trifasciata</i>		
	³⁵ S-labeled webs		RSR*	¹⁴ C-labeled webs		RSR†
	Mole%	NA		Mole%	RSR†	
Readily synthesized LMM						
Alanine	0.01±0.010	NA	0.05±0.027	–	–	88.2 (51.4–151) ^j
Glycine	0.53±0.306	NA	2.3±0.49	37.8 (27.1–52.9) ^d	–	40.2 (23.4–69.0) ^{hi}
GABamide	57.6±2.51	NA	53.9±1.89	19.6 (14.0–27.5) ^{c,d}	–	10.2 (5.97–17.6) ^{e,f,g}
Proline	ND	NA	ND	–	–	–
Moderately synthesized LMM						
<i>N</i> -Acetylputrescine	ND	NA	ND	–	–	3.53 (2.06–6.05) ^{c,d,e}
Putrescine moiety						2.70 (1.57–4.63) ^{c,d}
Acetyl moiety						5.05 (2.94–8.67) ^{d,e,f}
Putrescine	ND	NA	ND	–	–	–
Taurine	1.5±0.45	66.9 ^l (58.0–77.2) ^b	0.95±0.207	–	–	–
<i>N</i> -Acetyltaurine	12.6±0.66	52.9 (47.8–58.5) ^a	15.2±1.22	12.9 (9.19–18.0) ^c	14.1 (8.24–24.2) ^{f,g,h}	14.1 (8.24–24.2) ^{f,g,h}
Taurine moiety				2.24 (1.60–3.14) ^b	–	1.15 (0.67–1.97) ^{b,c}
Acetyl moiety				23.1 (16.5–32.3) ^{c,d}	–	27.0 (15.7–46.3) ^{g,hi}
Isethionic acid	8.3±1.07	47.1 (42.5–52.1) ^a	7.6±1.21	2.08 (1.49–2.91) ^b	–	0.69 (0.40–1.18) ^b
Poorly synthesized LMM						
Choline	15.7±2.00	NA	17.2±0.96	0.19 (0.14–0.27) ^a	–	0.16 (0.09–0.27) ^a
Glycine betaine	3.8±0.35	NA	2.8±0.23	–	–	–

See text for explanation of different categories of LMM (Readily, Moderately, and Poorly synthesized).

Web collections: *A. aurantia*, N=4 for both ³⁵S- and ¹⁴C-labeled webs; for *A. trifasciata*, N=3 web collections.

Molar percentages (Mole%, mean ± s.e.m.) in this table were calculated based on the 11 organic LMM constituting 100% of the LMM. Elsewhere, molar percentages were calculated based on the 11 organic LMM plus H₂PO₄⁻ constituting 100% of the LMM.

RSR, relative specific radioactivity (mean and 95% confidence interval). *For ³⁵S-labeled web extracts, specific radioactivities were normalized within each web collection by expressing the specific activity of each LMM as a percentage of the sum of the specific activities of NAT+Hse (c.p.m./mole S). †For ¹⁴C-labeled web extracts, normalized specific activities were similarly expressed as a percentage of the sum of the specific activities of Cho+GAB+Gly+NAT+Tau moiety of NAT+acetyl moiety of NAT+Hse (i.e. the constituents for which we have specific activity data in both *Argiope* species) (c.p.m./mole C). Thus, the mean specific activities reported from the ¹⁴C-labeled webs are directly comparable between the two species.

Within the webs of a single species labeled with a single radioisotope, specific radioactivities differed significantly (*P*<0.05) from each other if they show no superscript letters in common. See 'Statistical Analyses' in Materials and methods for more details.

NA, not applicable; ND, none detected by ¹H-NMR.

^lN=2 (taurine was not adequately resolved in the other two web collections).

of the Put component of NAP was measured. The specific activity of free Ala was determined only from the *A. trifasciata* web extracts because of the very small amounts of Ala in the *A. aurantia* extracts. Specific activities for Bet and ^{14}C -labeled Tau were not obtained because we were not convinced these compounds had been adequately resolved. However, relative specific activities were determined for ^{35}S -labeled Tau and for the Tau moiety of ^{14}C -labeled NAT.

In ^{14}C -labeled webs, the specific activity of NAT was significantly higher than that of Ise (Table 1). Hydrolysis of NAT, however, revealed that the bulk of the ^{14}C incorporated into this compound was restricted to its acetyl group and that the specific activities of Ise and the Tau moiety of NAT were not significantly different. Neither were the specific activities of NAT and Ise significantly different in ^{35}S -labeled webs. In contrast to NAT, the label in NAP was more evenly divided between the acetyl and Put moieties, though on a per mole carbon basis, the acetyl group's specific activity was again higher than that of Put, though not significantly so. This difference between the two *N*-acetylated compounds was in greater measure due to the significantly higher specific activity of the acetyl group in NAT as compared with the acetyl group in NAP, and to a lesser extent due to the higher (though not significantly so) specific activity of the Put moiety as compared with the Tau moiety. Recall that comparisons between these two LMM could be made only in *A. trifasciata* since NAP was not detected in *A. aurantia*.

Based on the specific radioactivities obtained, we divided the LMM in Table 1 into three categories: readily synthesized LMM (Ala, Gly, GAB), moderately synthesized LMM (NAP, Put, NAT, Tau, Ise), and poorly synthesized LMM (Cho, Bet). Assuming the acetyl moieties of NAP and NAT can be readily synthesized, LMM with mean specific activities greater than that of the acetyl group of NAP were taken to be readily synthesized. As we did not obtain specific activities for Pro, its placement is uncertain, but it is likely at least a moderately synthesized compound. Free Put and Tau were assumed to have specific activities comparable to those of the Put and Tau moieties of NAP and NAT, respectively, and it was the data for these moieties that dictated the inclusion of NAP and NAT in the moderately synthesized category. Though we also did not obtain data for Bet, we tentatively consider it a poorly synthesized LMM, given earlier observations (Higgins and Rankin, 1999) and the likelihood of its synthesis from Cho (see Discussion).

We predicted that, with starvation, molar percentages of readily synthesized LMM would tend to increase while those of poorly synthesized LMM would decrease and these trends would be reversed when feeding was resumed. The fate of moderately synthesized LMM was more uncertain, but our suspicion was that their molar percentages would either decrease or just be maintained with starvation.

Molar percentages of organic LMM were measured in the radiolabeled web collections simply to allow us to express radioisotope incorporation in terms of relative specific radioactivities. We assumed LMM compositions in these webs

would be similar to those in some of the non-radioactive web collections. In some respects they were, but there were also differences. The molar percentages of Cho and Bet are especially noteworthy in this regard. The lowest molar percentage of Cho in the eight radiolabeled *A. aurantia* web extracts (10.9 mole%) was higher than the highest molar percentage of Cho in the 102 unlabeled *A. aurantia* web extracts (8.9 mole%). (Note that these two molar percentages were calculated based on the 11 organic LMM constituting 100% of the LMM. The highest molar percentage for Cho in *A. aurantia* given in Table 2 is 7.4 mole% because the percentages in that table were calculated based on the 11 organic LMM plus H_2PO_4^- constituting 100% of the LMM.) Likewise, the lowest molar percentage of Cho in the three labeled *A. trifasciata* web extracts (22.6 mole%) was higher than the highest molar percentage of Cho in the 123 unlabeled *A. trifasciata* web extracts (13.9 mole%). The same sort of discrepancy, albeit not as extreme, was also observed in *A. aurantia* with regard to molar percentage of Bet. Unlabeled webs were built by adult female *Argiope* maintained on a diet of flies. They were collected as juveniles and raised in the laboratory through 1–2 molts and were unmated. Labeled webs were built by adult female *Argiope* that had recently been feeding in the field or, if fed in captivity, were given crickets or grasshoppers. They were collected as adults and were likely mated.

Effects of starvation and web removal on orb web mass and LMM composition

The number of webs composing each web collection is given in supplementary material Table S1.

Gravimetric data

Mass per orb web

Withholding prey and removing webs resulted in an immediate drop in mean orb web mass in four of the five starving groups and the downward trend continued until feeding was resumed (Fig. 1, supplementary material Figs S3–S5). Only in the *Argiope aurantia* starving adult group was there a slight delay before web mass likewise declined (supplementary material Fig. S6). When feeding was resumed, web mass quickly increased, though, among adults, *Argiope trifasciata* were slower to recover web mass than *A. aurantia* or *Araneus cavaticus*. Among *A. cavaticus*, juvenile males and females were slower to recover web mass than adult females. Web mass among resumed feeding adults, but not juveniles, ultimately returned to or exceeded the mass at the start of the stadium.

Among feeding group juvenile *A. cavaticus*, web mass was maintained or slightly increased in the earlier part of the stadium for both females and males. As they approached their final molt, web mass for females remained high (supplementary material Fig. S4), while those for males declined (supplementary material Fig. S3). Among feeding adult and *A. aurantia* PES groups, an upward trend followed by a decline in the days leading up to egg sac construction was

typical (Fig. 1, supplementary material Figs S5–S7). In the *A. trifasciata* PES groups, an initial upward trend was less in evidence, but decreases in web mass prior to construction of the next egg sac were observed (supplementary material Fig. S7). Web mass in the three resumed feeding adult groups, however, did not tend to drop near the end of the series (Fig. 1, supplementary material Figs S5, S6).

Web mass as a percentage of spider mass

Table 3 presents data on fresh masses of the three species of spiders and their dry masses fractions. It also presents desiccated masses of webs built by feeding group spiders and the total masses of the 12 measured LMM in these webs. From these data we estimate that these spiders typically invested about 1–3% of their dry mass into the materials composing an orb web and about 0.5–1% of their dry mass into the 12 measured LMM per web.

Water-soluble percentages

For *A. cavaticus*, the percentage of the desiccated web mass extracted by water (mean \pm s.d.) was $71.0 \pm 4.24\%$, $N=86$ web collections (juveniles $72.4 \pm 4.06\%$, $N=51$; adults $68.9 \pm 3.57\%$, $N=35$). For *A. aurantia* and *A. trifasciata*, as observed previously (Townley et al., 1991), water-soluble percentages were usually lower, means \pm s.d. $54.6 \pm 5.70\%$ ($N=105$) and $52.8 \pm 7.45\%$ ($N=123$), respectively. Water-soluble fractions undoubtedly include some non-LMM components, such as nodular glycoprotein (see Introduction). Very low water-soluble percentages, far below the averages, were seen in some web collections built at or near the end of the series in the *A.*

trifasciata feeding, resumed feeding, PES2 and PES3 groups, and in the *A. aurantia* PES1 group. It is likely that additional examples of very low water-soluble percentages were masked as a result of end-of-series ‘horizontal’ pooling (see ‘Gravimetric measurements of orb webs’ in Materials and methods and supplementary material Table S1).

Water-soluble percentages tended to decrease over the series. Slopes of linear regressions differed significantly from zero in 14 of the 21 groups, and these 14 slopes were all negative (range: -0.22 to -1.65) and spread across all species and treatments (supplementary material Table S3).

The percentage of the water-soluble mass accounted for by the 11 organic LMM in *A. cavaticus* was $57.8 \pm 5.4\%$ (mean \pm s.d., $N=86$). There was a tendency for this percentage to decrease over the series irrespective of treatment, but linear regression slopes departed significantly from zero in only three of the nine groups of *A. cavaticus* (feeding juvenile females, $P=0.005$; starving adult females, $P=0.030$; resumed feeding adult females, $P=0.030$). When the data from all nine groups were pooled, the slope of the regression was significantly different from zero ($P=0.039$), with an average decrease from one web collection to the next of 0.27%.

LMM compositional data

Molar percentage data (averages and ranges) for the 11 organic LMM and inorganic phosphate in the non-radioactive webs of each species are summarized in Table 2. These data were also analyzed for correlations between LMM (Table 4). Some significant correlations showed agreement among our

Table 2. Relative quantities (in mole%) of the organic LMM and inorganic phosphate in orb webs of the three species studied, as measured in non-radioactive web collections

	<i>Araneus cavaticus</i>		<i>Argiope aurantia</i>		<i>Argiope trifasciata</i>	
	Mean ¹ \pm s.e.m.	Range ²	Mean ¹ \pm s.e.m.	Range ²	Mean \pm s.e.m. ¹	Range ²
Putrescine	0.58 ± 0.067	ND–1.24	ND	ND	1.11 ± 0.157	ND–1.85
Choline	7.5 ± 0.94	0.3–15.9	4.2 ± 0.90	0.2–7.4	6.7 ± 1.28	ND–12.2
GABamide	42.1 ± 1.05	31.6–58.3	52.6 ± 1.73	47.6–70.8	3.9 ± 0.93	0.6–31.8
<i>N</i> -acetylputrescine	3.13 ± 0.186	ND–6.60	ND	ND	32.8 ± 0.97	14.7–43.0
Glycine betaine	0.37 ± 0.083	ND–2.42	0.65 ± 0.227	ND–2.00	2.4 ± 0.61	ND–5.0
Glycine	1.33 ± 0.157	0.25–15.5	2.5 ± 0.55	0.7–19.9	8.9 ± 0.55	2.6–31.6
Alanine	0.45 ± 0.043	ND–2.76	0.16 ± 0.030	ND–2.85	2.82 ± 0.207	0.18–6.06
Proline	0.76 ± 0.159	ND–8.83	ND	ND–1.65	ND	ND
Taurine	ND	ND	0.36 ± 0.048	ND–0.58	0.32 ± 0.101	ND–0.80
<i>N</i> -acetyltaurine	6.0 ± 0.50	2.0–14.4	12.7 ± 0.64	2.5–17.2	10.3 ± 1.25	0.2–13.2
Isethionate	24.5 ± 0.75	6.0–33.1	8.8 ± 0.77	1.5–11.6	19.1 ± 1.71	9.2–39.8
H ₂ PO ₄ ⁻	13.2 ± 0.37	10.0–22.7	18.1 ± 0.46	8.7–22.8	11.6 ± 0.28	4.8–15.8

All inorganic phosphorus was assumed to be present as H₂PO₄⁻.

ND, none detected by ¹H-NMR.

N values: *Araneus cavaticus*: means, $N=21$; ranges, $N=86$; *Argiope aurantia*: means, $N=7$; ranges, $N=102$; *Argiope trifasciata*: means, $N=7$; ranges, $N=123$.

¹Means were calculated using only data from web collections 2–6 from feeding groups and web collections 2–3 from starving groups. This selection is somewhat arbitrary, but averages calculated from these data are probably closer to typical molar percentages in field-built webs than are means calculated using data from all web collections. Even so, some of the means presented (e.g. Cho) may still deviate substantially from average field-built webs.

²Ranges consider data from all non-radioactive web collections.

three species and/or with *Nephila clavipes* L. (Higgins et al., 2001) and these are indicated in Table 4.

Changes in molar percentages of eight of the organic LMM with starvation and subsequent resumed feeding are shown in Figs 2–4. Fig. 2 contains the readily synthesized LMM, Ala, Gly, and GAB, Fig. 3 the three most abundant moderately synthesized LMM, NAP, NAT and Ise, and Fig. 4 the poorly synthesized LMM, Cho and Bet (see ‘Synthesis of organic LMM by *Argiope*’ above). These figures also present the corresponding data from the feeding groups.

Bar charts showing both the absolute and relative quantities of all 12 measured LMM in web collections from the *A. trifasciata* starving, resumed feeding, and feeding groups are presented in Fig. 1. Supplementary material Figs S3–S6 show the corresponding bar charts for *A. cavaticus* and *A. aurantia* (key in supplementary material Fig. S2). Absolute and relative quantity data for the 12 LMM in each web collection from all groups are also available in tabular form in supplementary material Tables S4–S6.

In all tables and figures except Table 1, molar percentages were calculated based on the 12 LMM constituting 100% of the LMM. (In Table 1, molar percentage was calculated based on the 11 organic LMM constituting 100% of the LMM.) In all three species, however, several as-yet-unidentified and generally minor organic LMM were detected in many of the spectra. There are undoubtedly other inorganic LMM components as well, such as NO_3^- , K^+ , Na^+ and Ca^{2+} reported from webs of other araneoid species (Schildknecht et al., 1972; Work, 1981; Tillinghast and Christenson, 1984; Patel and Nigam, 1996).

Organic LMM

We predicted that molar percentages of Ala, Gly and GAB would increase with starvation and then drop when feeding was resumed. Conforming to expectations, percentages of Gly and GAB increased with starvation in all three species, at least in the earlier part of the series (Fig. 2). It is especially noteworthy that not only molar percentages, but also absolute quantities of

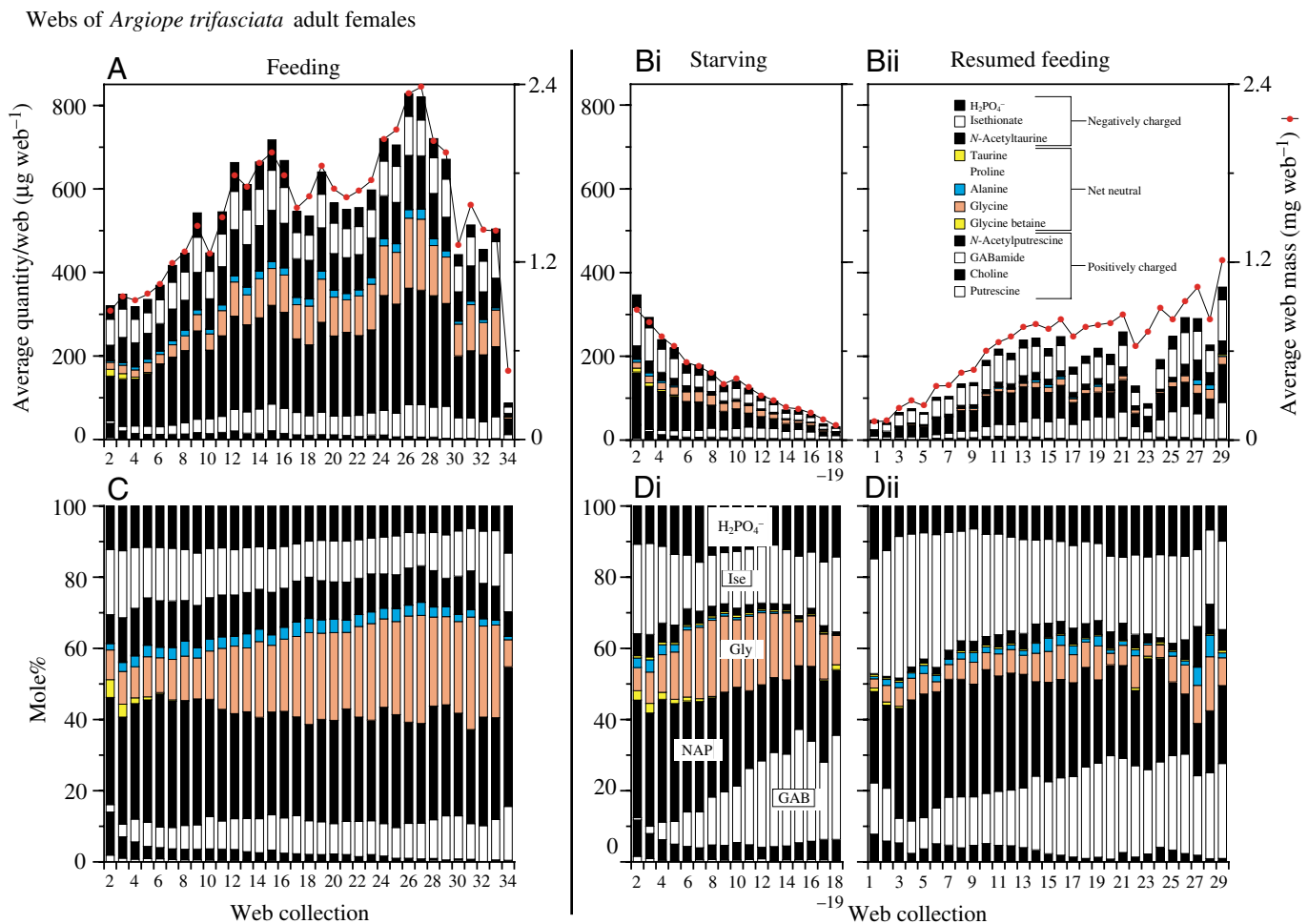


Fig. 1. Absolute (A,B) and relative (C,D) quantities of LMM in water extracts of webs built by *Argiope trifasciata* adult females in feeding (A,C) and starving/resumed feeding (B,D) groups. Average desiccated web masses within web collections are indicated by line graphs in A and B (right y-axis). The key (Bii) identifies the LMM, arranged in the same order, represented in all six bar charts. Several major LMM are also directly labeled in Di to further aid orientation. LMM with a net charge are represented by solid black or white, while net neutral LMM are shown in color. Proline was not detected in any *A. trifasciata* web collections. GAB, GABamide; Gly, glycine; Ise, isethionate; NAP, N-acetylputrescine.

Table 3. *Spider and feeding group orb web mass data*

Species	Instar	Sex	Spider				Feeding group			
			Fresh mass (mg)	<i>N</i>	Dry mass/fresh mass	<i>N</i>	Web mass (mg)	<i>N</i>	Mass of LMM (mg)	<i>N</i>
<i>A. cavaticus</i>	Pen.	M					1.05±0.028 (web collections 7–10 excluded)	5	0.52±0.025	5
<i>A. cavaticus</i>	Pen.	F	327±18.5	22	0.270±0.009	7	1.18±0.037	10	0.56±0.023	10
<i>A. cavaticus</i>	Adult	F	518±62.6	11	0.268±0.008	4	1.59±0.041 (web collections 17–19 excluded)	15	0.74±0.020	15
<i>A. aurantia</i>	Adult	F	315±41.1	7	0.313±0.026	7	1.89±0.073 (web collections 27–29 excluded)	25	0.74±0.030	25
<i>A. trifasciata</i>	Adult	F	265±16.7	12	0.265±0.011	6	1.59±0.070 (web collection 34 excluded)	32	0.56±0.025	32

F=female; M=male; Pen.=penultimate instar; LMM=low-molecular-mass compounds (includes 11 organic compounds listed in Introduction plus H₂PO₄⁻).

Values are means ± s.e.m.; *N*=number of observations.

Spider masses for penultimate instar male *A. cavaticus* were not obtained but are probably comparable to those of penultimate instar females. Note that some of the variation in spider mass in all three species may be attributable to variation in the number of stadia spiders pass through before arriving at the penultimate instar and, consequently, variation also in the number of stadia preceding adulthood.

Feeding group desiccated web mass and total mass for the 12 measured LMM are presented here to provide an indication of values that are typical for these spiders, at least in the laboratory, when they are capturing prey (but not allowed to recycle their webs beyond the first post-ecdysial web). Webs with especially low mass at the end of a series have been excluded from the presented means since they are viewed as being more atypical, compromised webs resulting from internal preparations for molting or egg laying.

Gly and GAB increased in the webs of starving spiders; Gly in all three species and GAB in *Argiope trifasciata*, again, at least in the first portion of the series (Fig. 1, supplementary material Figs S3–S6 and Tables S4–S6). With resumed feeding, molar percentages of Gly immediately dropped in all three species, as did GAB in *A. trifasciata*, though, unexpectedly, they again tended to increase as the resumed feeding series progressed. Other trends were also not expected: free Ala remained a minor component in starving *Araneus cavaticus* and *Argiope aurantia*, increasing only slightly (indeed, less than in feeding groups), and in *A. trifasciata* Ala clearly declined with starvation (Fig. 2). Also, GAB did not decrease with resumed feeding in *A. cavaticus* and *A. aurantia*. Even more unanticipated, increases in Gly and GAB were also seen in the feeding groups, where more stable compositions had been expected. These increases are reflected in positive correlations in feeding groups between web collection number and the summed molar percentages of the three readily synthesized LMM, very similar to correlations seen in the starving groups (Table 5).

We suspected that the moderately synthesized LMM would decrease or at most be maintained in starving spiders. The low specific activities of Ise and NAT (Table 1) made it especially likely that they would decline. With starvation we observed trends toward decreased molar percentages of not only Ise and NAT, but NAP as well (Fig. 3). In starving *A. cavaticus* females, however, decreases in NAT were preceded by increases such that percentages of NAT at the end of the series were similar to those at the beginning. Starving spiders

responded to resumed feeding with increased molar percentages of NAP, NAT and Ise, though subsequent trends varied as the resumed feeding series progressed and include examples of declines in molar percentages after the initial rise (e.g. Ise in all three species) as well as more prolonged upward trends (e.g. NAT in *A. trifasciata* and *A. cavaticus*). Interestingly, in contrast to the rapid and substantial recovery of Ise in resumed feeding *A. trifasciata*, resulting in high molar percentages of Ise relative to feeding group *A. trifasciata*, NAT's recovery was slow and modest, resulting in low percentages of NAT relative to the feeding group (Figs 1, 3).

In agreement with expectations, molar percentages of NAP were essentially maintained by feeding spiders and, while not necessarily maintained at a constant level, percentages of NAT in webs of feeding spiders were generally higher than in the corresponding webs of starving spiders. Ise, however, exhibited molar percentage declines in webs of feeding spiders that were sometimes very similar to those of starving spiders. These contributed to negative correlations between web collection number and summed molar percentages of the moderately synthesized LMM in feeding groups, though these correlations were less significant than those of the starving groups (Table 5). Negative correlations between total readily synthesized and moderately synthesized LMM, and positive correlations between moderately synthesized and poorly synthesized LMM, in both starving and feeding groups, indicate that the moderately synthesized LMM generally exhibit shifts in relative abundance more like poorly synthesized, rather than readily synthesized, LMM (Table 5).

Table 4. Pearson correlation matrices for arcsine-transformed molar percentages of the 12 LMM in all non-radioactive web collections from each species

		Put	Cho	GAB	NAP	Bet	Gly	Ala	Pro	Tau	NAT	Ise
Cho	<i>A. cav.</i>	<u>0.454</u> ***										
	<i>A. aur.</i>	–										
	<i>A. tri.</i>	0.366 **										
GAB	<i>A. cav.</i>	<u>–0.604</u> ***	–0.745 ***									
	<i>A. aur.</i>	–	–0.272									
	<i>A. tri.</i>	<u>–0.470</u> ***	–0.149									
NAP	<i>A. cav.</i>	0.662***	0.080	<u>–0.497</u> ***								
	<i>A. aur.</i>	–	–	–								
	<i>A. tri.</i>	0.020	–0.099	<u>–0.519</u> ***								
Bet	<i>A. cav.</i>	0.346	0.558 ***	<u>–0.574</u> ***	0.298							
	<i>A. aur.</i>	–	0.828 ***	<u>–0.397</u> **	–							
	<i>A. tri.</i>	0.364**	0.513 ***	<u>–0.356</u> **	0.046							
Gly	<i>A. cav.</i>	–0.615 ***	–0.386*	0.372 *	–0.677***	–0.172						
	<i>A. aur.</i>	–	–0.283	–0.304	–	–0.197						
	<i>A. tri.</i>	0.359**	<u>–0.437</u> ***	–0.303*	–0.198	–0.247						
Ala	<i>A. cav.</i>	<u>–0.462</u> ***	<u>–0.647</u> ***	0.426**	–0.147	–0.408**	0.457 ***					
	<i>A. aur.</i>	–	<u>–0.480</u> ***	–0.021	–	–0.335*	0.598 ***					
	<i>A. tri.</i>	0.096	–0.293	<u>–0.544</u> ***	0.077	–0.139	0.459 ***					
Pro	<i>A. cav.</i>	<u>–0.489</u> ***	<u>–0.494</u> ***	0.256	–0.421**	–0.195	<u>0.834</u> ***	<u>0.546</u> ***				
	<i>A. aur.</i>	–	<u>–0.377</u> **	–0.143	–	–0.275	<u>0.456</u> ***	<u>0.650</u> ***				
	<i>A. tri.</i>	–	–	–	–	–	–	–				
Tau	<i>A. cav.</i>	–	–	–	–	–	–	–	–			
	<i>A. aur.</i>	–	<u>0.504</u> ***	–0.223	–	<u>0.325</u> *	–0.112	<u>–0.356</u> *	–0.182			
	<i>A. tri.</i>	–0.027	<u>0.640</u> ***	0.257	–0.160	<u>0.307</u> *	<u>–0.598</u> ***	<u>–0.512</u> ***	–			
NAT	<i>A. cav.</i>	0.038	<u>–0.413</u> **	–0.061	0.552***	–0.006	–0.068	0.498***	0.209	–		
	<i>A. aur.</i>	–	<u>–0.339</u> *	<u>–0.412</u> ***	–	–0.150	0.006	0.280	0.143	–0.129		
	<i>A. tri.</i>	0.312*	<u>–0.459</u> ***	<u>–0.508</u> ***	0.113	–0.094	0.615***	0.754***	–	–0.722***		
Ise	<i>A. cav.</i>	0.695***	0.534***	<u>–0.637</u> ***	0.715***	0.395**	<u>–0.871</u> ***	<u>–0.588</u> ***	<u>–0.735</u> ***	–	0.025	
	<i>A. aur.</i>	–	0.258	–0.329*	–	0.246	<u>–0.554</u> ***	<u>–0.535</u> ***	<u>–0.395</u> **	0.277	0.253	
	<i>A. tri.</i>	<u>–0.454</u> ***	0.396***	0.203	0.130	0.143	<u>–0.838</u> ***	<u>–0.369</u> **	–	0.664***	<u>–0.730</u> ***	
PO ₄ [–]	<i>A. cav.</i>	<u>–0.376</u> *	–0.025	0.247	<u>–0.539</u> ***	–0.099	0.360*	–0.030	0.171	–	<u>–0.521</u> ***	<u>–0.469</u> ***
	<i>A. aur.</i>	–	0.342*	–0.082	–	0.244	<u>–0.466</u> ***	<u>–0.600</u> ***	<u>–0.323</u> *	0.172	<u>–0.451</u> ***	0.198
	<i>A. tri.</i>	0.179	0.424***	0.259	<u>–0.326</u> *	0.404***	<u>–0.296</u> *	<u>–0.418</u> ***	–	0.335**	–0.190	–0.065

A. cav., *A. cavaticus* (N=86); *A. aur.*, *A. aurantia* (N=102); *A. tri.*, *A. trifasciata* (N=123).

Ala, alanine; Bet, glycine betaine; Cho, choline; GAB, GABamide; Gly, glycine; Ise, isethionate; NAP, *N*-acetylputrescine; NAT, *N*-acetyltaurine; Pro, proline; Put, putrescine; Tau, taurine.

Bonferroni probabilities: * $P \leq 0.05$; ** $P < 0.01$; *** $P \leq 0.001$.

Missing correlation coefficients indicate LMM not detected in webs of that species. Underlined coefficients indicate significant correlations that agree among the three species (i.e. all three negative or all three positive), or between two species if either of the LMM being compared went undetected in the third species. Coefficients in bold indicate significant correlations that agree with significant correlations seen in webs of *N. clavipes* (Higgins et al., 2001). In that study, NAP and Ise were not detected in web washes, H₂PO₄[–] was not assayed, and Pro and Tau, while detected in some web washes, were not included in statistical analyses.

Several correlations between NAT and other LMM (Table 4), however, demonstrate that this is not invariably the case (though Table 4, unlike Table 5, considers data from resumed feeding and PES groups as well as from starving and feeding groups).

Cho and Bet were expected to decline with starvation and, indeed, declines were observed in all three species (Fig. 4). But

as with GAB, Gly, and Ise, similar trends were also unexpectedly exhibited by the feeding groups (Table 5). Moreover, in the starving groups, after a steep initial decline, Cho tended to level off (or partially rebound) at molar percentages that exceeded the percentages of Cho in the corresponding feeding group webs. In some instances, even $\mu\text{g Cho web}^{-1}$ was greater in starving groups than in the

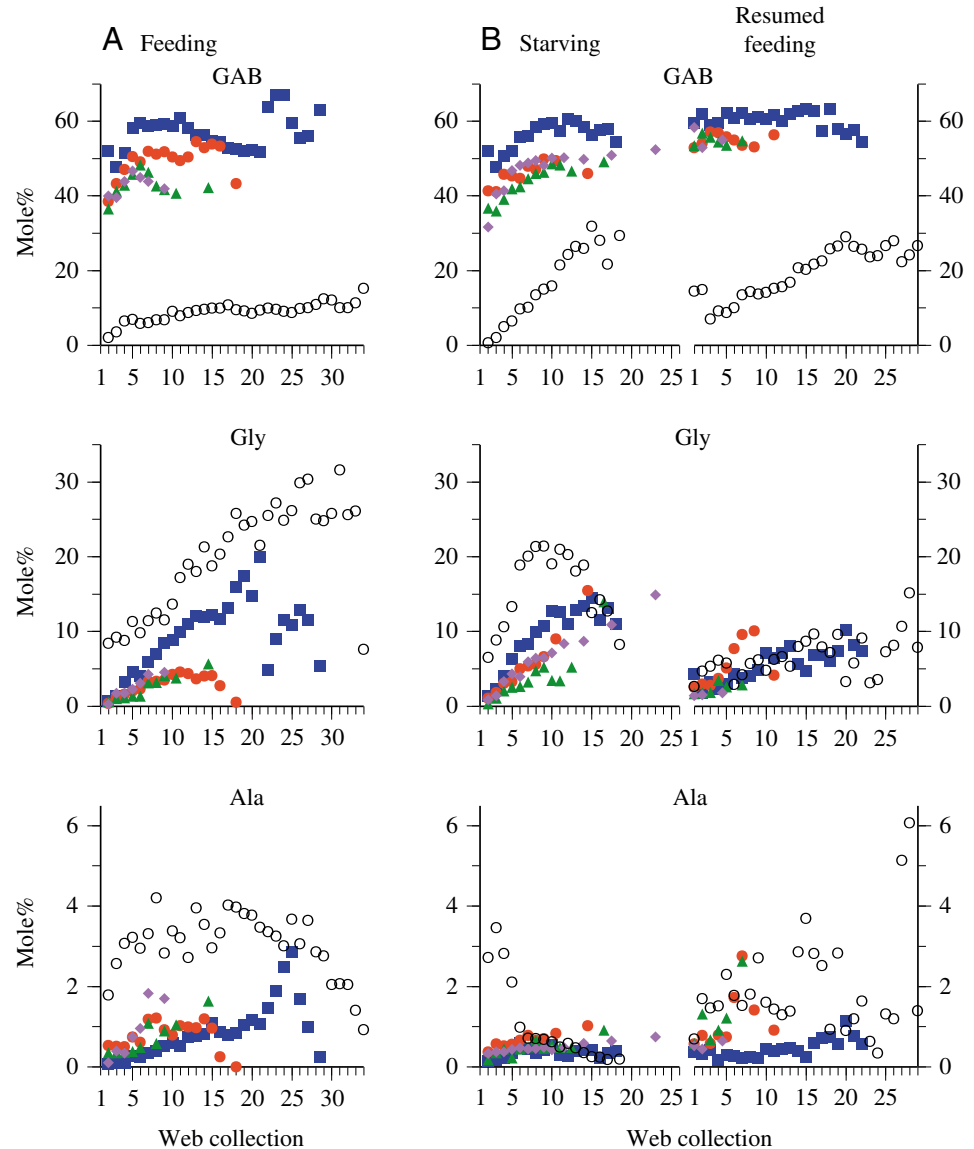


Fig. 2. Molar percentages of GABamide (GAB), free glycine (Gly) and free alanine (Ala) in water extracts of webs built by (A) feeding and (B) starving/resumed feeding groups. In this and Figs 3, 4, a data point representing horizontally pooled web collections, e.g. web collections 14–19, was assigned an x -coordinate midway in this range, i.e. web collection 16.5 (see Materials and methods for explanation of ‘horizontal pooling’ and supplementary material Table S1). Note the very different molar percentage ranges for different compounds. Purple diamonds, juvenile male *Araneus cavaticus*; green triangles, juvenile female *A. cavaticus*; red circles, adult female *A. cavaticus*; blue squares, adult female *Argiope aurantia*; open circles, adult female *Argiope trifasciata*.

corresponding feeding group webs (supplementary material Figs S3–S6 and Tables S4, S5). This contrasted with what we generally observed: greater absolute quantities of LMM per web (often considerably so) in feeding groups than in starving groups due to the usually greater total web mass (Fig. 1, supplementary material Figs S3–S6) and mass of water-soluble fractions in feeding groups. In both *Argiope* species, negative correlations between web collection number and Cho + Bet were not significant in starving groups (Table 5) primarily due to unexpected end-of-series increases in Cho and Bet. In starving *A. cavaticus* groups (juveniles especially), Bet molar percentage decreases occurred only after initial unanticipated increases (Fig. 4). Comparable increases were not seen in feeding *A. cavaticus*. Also contrary to expectations, resumed feeding of starving spiders yielded little or no resurgence in Cho and Bet.

Of the remaining three organic LMM measured in this study, Put and Tau were invariably minor components (<2 mole%)

and free Pro's contribution exceeded 2 mole% only in some *A. cavaticus* webs (Table 2). Each went undetected in all webs from one of the three study species, though it may be that small quantities were not detected because of overlapping peaks from other compounds, including minor unidentified compounds. It now appears that the compound identified as Tau in earlier analyses of *A. cavaticus* webs (Townley et al., 1991) was not Tau but a minor as yet unidentified compound. Shifts in Pro were reminiscent of Gly with upward trends in both starving and feeding *A. cavaticus*, and a drop when feeding was resumed by starving spiders (supplementary material Figs S3–S5). Absolute quantities of Pro per web were generally maintained or increased in starving *A. cavaticus* (supplementary material Table S4).

Post-egg-sac (PES) webs

The construction of up to three egg sacs by some *Argiope aurantia* and *Argiope trifasciata* and the building of orb webs

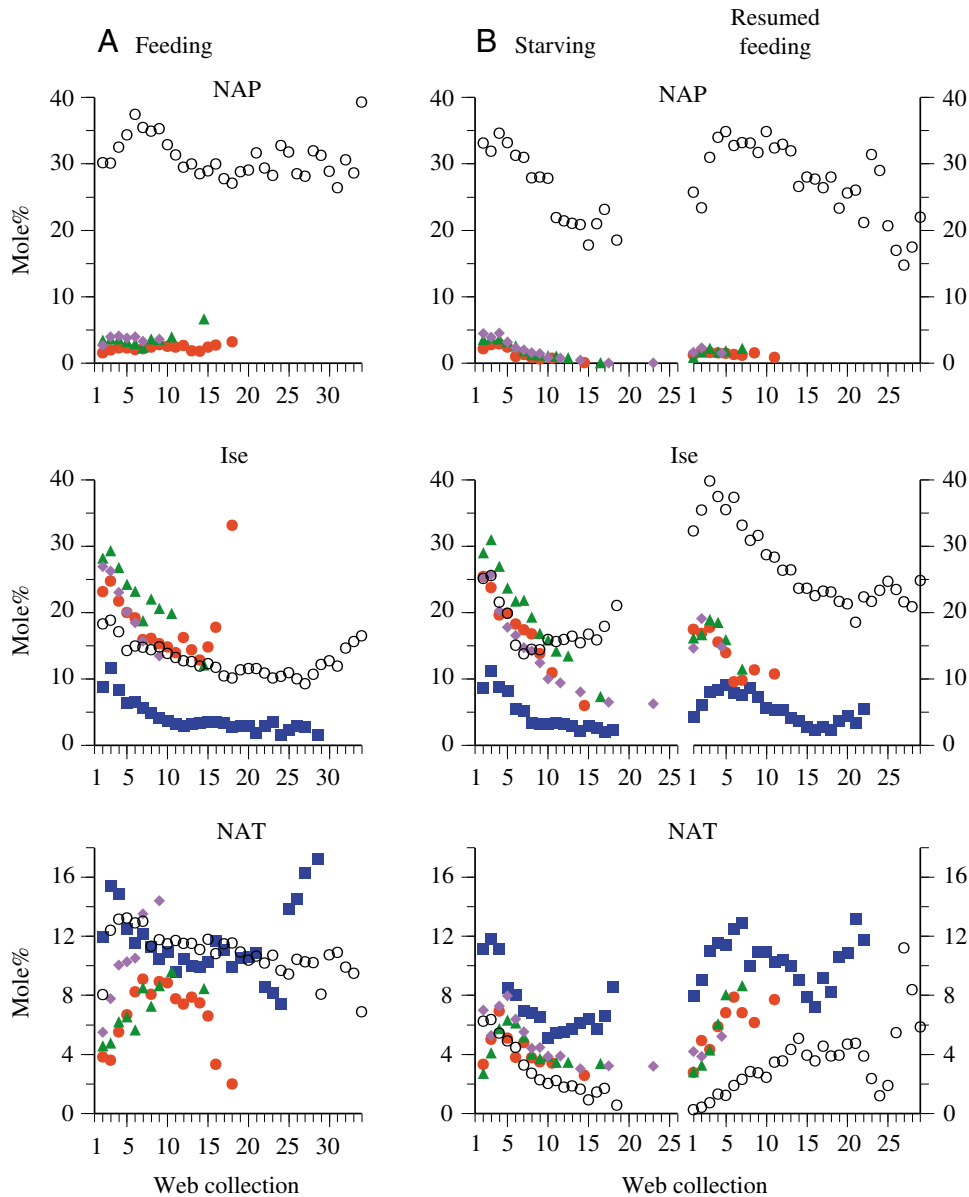


Fig. 3. Molar percentages of *N*-acetylputrescine (NAP), isethionate (Ise), and *N*-acetyltaurine (NAT) in water extracts of webs built by (A) feeding and (B) starving/resumed feeding groups. Purple diamonds, juvenile male *Araneus cavaticus*; green triangles, juvenile female *A. cavaticus*; red circles, adult female *A. cavaticus*; blue squares, adult female *Argiope aurantia*; open circles, adult female *Argiope trifasciata*.

after each of these gave us the opportunity to examine changes in LMM composition in webs built between egg laying episodes by feeding spiders (though, as throughout this study, determining LMM composition meant that spiders were not allowed to recycle webs). Cyclical changes in LMM molar percentages were evident when the data from successive PES groups were examined (Fig. 5; absolute quantities of LMM shown in supplementary material Fig. S7). To mention just two examples, in both species Gly was relatively low following egg sac construction, but increased subsequently and then declined with the approach of the next oviposition. Cho, on the other hand, was highest in the first webs built after an egg sac was made and tended to decline as the series progressed.

$H_2PO_4^-$ and charge balance

In the bar charts in Figs 1, 5 and supplementary material

Figs S3–S7, LMM carrying a net positive charge at the pH of the sticky droplets [about 4 in *Araneus diadematus* Clerck 1757 (Schildknecht et al., 1972); 7.9 in *Argiope anasuja* Thorell 1887 (Patel and Nigam, 1996)] are grouped together, as are net negative and net neutral LMM.

When the molar percentages of the positively charged organic LMM (Put, Cho, GAB and NAP) were added (doubling Put because of its +2 charge) and compared with the sums of the negative organic LMM (NAT and Ise) there was an 'excess' of positive charge in all 311 web collections analyzed during this study. This excess was greatest in *Argiope aurantia* (43.6 ± 0.70 mole%, mean \pm s.e.m.; $N=102$) and least in *Argiope trifasciata* (21.5 ± 0.65 mole%; $N=123$), with *Araneus cavaticus* intermediate (31.0 ± 0.86 mole%; $N=86$). Analyzing the data from each group separately revealed a highly significant ($P \leq 0.001$) increase over the series in the

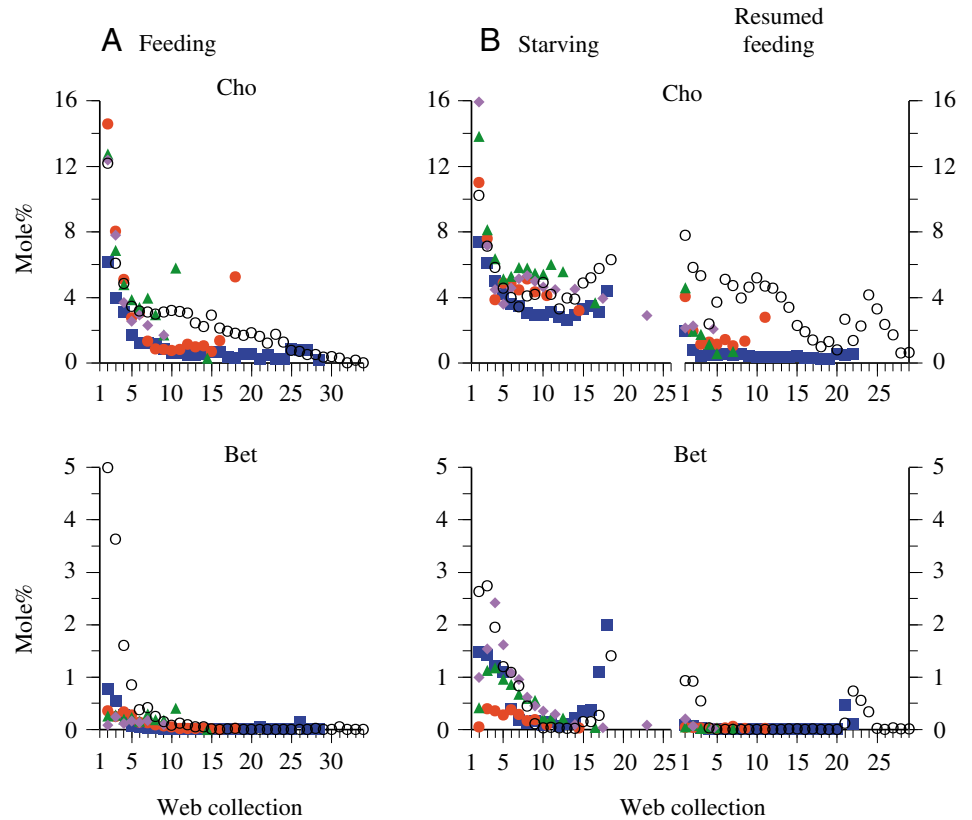


Fig. 4. Molar percentages of choline (Cho) and glycine betaine (Bet) in water extracts of webs built by (A) feeding and (B) starving/resumed feeding groups. Purple diamonds, juvenile male *Araneus cavaticus*; green triangles, juvenile female *A. cavaticus*; red circles, adult female *A. cavaticus*; blue squares, adult female *Argiope aurantia*; open circles, adult female *Argiope trifasciata*.

excess positive charge in all five starving groups and these increases were greater than and significantly different ($P \leq 0.05$) from increases seen in any other groups. In *A. cavaticus*, there was no significant difference between the starving adult group and either of the starving juvenile groups. Much of the increase in excess positive charge in the starving groups was due to a drop in negatively charged organic LMM (NAT+Ise) over the series rather than to an increase in positively charged organic LMM. In the three starving *A. cavaticus* groups, >91% of the increased excess positive charge was due to a decrease in NAT+Ise. For the *A. aurantia* and *A. trifasciata* starving groups, this percentage was 74% and 48%, respectively.

All web extracts were assayed for inorganic phosphorus to estimate inorganic phosphate's contribution as a counterion to the excess positive charge. In only 9 of the 311 web collections did the molar percentage of H_2PO_4^- essentially balance or even slightly exceed the molar percentage of excess positive charge (six of the nine were in the *A. trifasciata* resumed feeding series and were due primarily to the relatively rapid recovery of Ise near the start of the series decreasing the excess positive charge). In the remaining 302 web collections the molar percentage of H_2PO_4^- was insufficient to balance the molar percentage of excess positive charge. On average, the percentage of the excess positive charge balanced by inorganic phosphate was least in the species with the greatest excess positive charge, *A. aurantia* (36.3%), and about equal in the other two species (*A. trifasciata* 47.7%, *A. cavaticus* 48.8%).

As noted above, the 12 LMM quantitated in this study do not provide a complete inventory of the LMM, especially of inorganic ions. And a more complete accounting of charges in this system should also consider components other than LMM, such as the nodular glycoprotein (see Introduction).

There was a positive correlation between the molar percentages of H_2PO_4^- and excess positive charge when the data from all three species were pooled ($r=0.651$, $P<0.001$, $N=311$), but only *A. cavaticus* ($r=0.546$, $P<0.001$, $N=86$) and *A. trifasciata* ($r=0.386$, $P<0.001$, $N=123$) showed significant correlations when each species was analyzed separately. In *A. cavaticus*, this correlation was attributable to a negative correlation between H_2PO_4^- and negatively charged organic LMM (NAT+Ise) ($r=-0.643$, $P<0.001$), as opposed to the situation in *A. trifasciata* in which there were significant correlations between H_2PO_4^- and both negatively charged ($r=-0.235$, $P=0.009$) and positively charged ($r=0.274$, $P=0.002$) organic LMM. The inverse relationship between H_2PO_4^- and the two sulfonic acids was mostly clearly exhibited in juvenile *A. cavaticus* (males and females) and contributed to significant inverse correlations between H_2PO_4^- and both NAT and Ise in *A. cavaticus* (Table 4).

Discussion

Synthesis of sticky droplet organic LMM

Radiolabeled glucose and methionine/cysteine were fed to spiders to assess their ability to synthesize sticky droplet

Table 5. Pearson correlation matrices describing relationships among web collection number and arcsine-transformed summed molar percentages of readily synthesized, moderately synthesized and poorly synthesized LMM in the starving and feeding groups of each species

			Web number	RS	MS
RS	Starving	<i>A. cavaticus</i>	0.883***		
		<i>A. aurantia</i>	0.773**		
		<i>A. trifasciata</i>	0.815***		
	Feeding	<i>A. cavaticus</i>	0.606**		
		<i>A. aurantia</i>	0.727***		
		<i>A. trifasciata</i>	0.808***		
MS	Starving	<i>A. cavaticus</i>	-0.926***	-0.947***	
		<i>A. aurantia</i>	-0.821***	-0.987***	
		<i>A. trifasciata</i>	-0.888***	-0.982***	
	Feeding	<i>A. cavaticus</i>	-0.533*	-0.841***	
		<i>A. aurantia</i>	-0.498	-0.880***	
		<i>A. trifasciata</i>	-0.609**	-0.898***	
PS	Starving	<i>A. cavaticus</i>	-0.702***	-0.873***	0.748***
		<i>A. aurantia</i>	-0.476	-0.888***	0.848***
		<i>A. trifasciata</i>	-0.444	-0.846***	0.752**
	Feeding	<i>A. cavaticus</i>	-0.688***	-0.874***	0.645***
		<i>A. aurantia</i>	-0.700***	-0.876***	0.789***
		<i>A. trifasciata</i>	-0.906***	-0.781***	0.466

RS, readily synthesised (GABamide, glycine, alanine); MS, moderately synthesized (*N*-acetylputrescine, *N*-acetyltaurine, isethionate, putrescine, taurine); PS, poorly synthesized (choline, glycine betaine) LMM.

Molar percentage data from adult female, juvenile female, and juvenile male starving and feeding groups were used to calculate *A. cavaticus* correlation coefficients. Coefficients for both *Argiope* species were calculated using molar percentage data from adult female starving and feeding groups.

Bonferroni probabilities: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

LMM. Label from [^{14}C]glucose appeared in high specific radioactivity in web droplet Ala, Gly and GAB, while only a meager amount was associated with Cho (Table 1), indicating that Cho is nutritionally essential. Ise, Tau (free and as a component of NAT) and the Put moiety of NAP occupied positions between these extremes, with the relatively low specific activities of the two sulfonic acids in particular raising the possibility that, without adequate dietary intake of these LMM (or more immediate precursors), the spider's synthetic rate may not always be sufficient to meet the requirements for optimal web construction. These results are essentially in agreement with the literature, as detailed in the following sections.

GABamide, glycine and alanine (readily synthesized LMM)

Relatively high specific activities for Ala, Gly and GAB are in keeping with previous studies on *Araneus cavaticus* fed [^{14}C]glucose (Townley and Tillinghast, 1988) and *Nephila clavipes* fed [^{14}C]glucose or [^{14}C]acetate (Higgins and Rankin, 1999). That Gly and Ala were readily synthesized is not surprising as these amino acids are generally dispensable in arthropods (Dall and Moriarty, 1983; Dadd, 1985; Guillaume, 1997). Nor was it surprising that in each of the three ^{14}C -

labeled *Argiope trifasciata* web collections the specific activity of Ala was higher than that of Gly. Such a disparity in animals administered [$\text{U-}^{14}\text{C}$]glucose has often been observed, including in other arachnids (Rodriguez and Hampton, 1966; Rodriguez and Lasheen, 1971), insects (see references in Kasting and McGinnis, 1966; also Rock and Hodgson, 1971; Widmer, 1973) and crustaceans (e.g. Shewbart et al., 1972; van Marrewijk and Zandee, 1975; Lasser and Allen, 1976; Miyajima et al., 1976).

In an earlier study with *N. clavipes* webs (Higgins et al., 2001), molar percentages of free Gly and Ala tended to be positively correlated. Likewise, in this study, Gly and Ala were positively correlated in the three species and, in *A. cavaticus* and *Argiope aurantia*, Pro was positively correlated with both Gly and Ala (Table 4) (Pro was not detected in webs of *A. trifasciata*).

N-Acetyltaurine, isethionate and *N*-acetylputrescine (moderately synthesized LMM)

Earlier studies examined the incorporation of radioisotope into LMM from webs built by *Argiope* fed [^{14}C]Tau or [^{35}S]cysteine (Anderson and Tillinghast, 1980; Tillinghast, 1984). However, as those studies preceded the identifications

of NAT and Ise (Vollrath et al., 1990), only tentative conclusions may be drawn from them. Nevertheless, it appeared that, after feeding either radioactive precursor, labeled NAT ('compound I' of Anderson and Tillinghast, 1980), Ise (consistent with 'compound II'), and Tau were present in webs built by *A. trifasciata*, while in *A. aurantia* only NAT was unambiguously labeled. This difference may have been due to different relative quantities of sulfonates on the webs: *A. aurantia* webs typically contain lower molar percentages of Ise and higher NAT than *A. trifasciata* webs (Vollrath et al., 1990; Townley et al., 1991) (Table 2). In webs of *N. clavipes*, Ise has not been detected by NMR (Higgins et al., 2001), but radiolabeling of NAT by spiders fed [^{14}C]glucose or [^{14}C]acetate has been observed, albeit inconsistently (Higgins and Rankin, 1999). Results obtained to date from *Argiope* fed [^{35}S]cysteine (Anderson and Tillinghast, 1980) (Table 1) indicate that these spiders can, like many other animals (Jacobsen and Smith, 1968; Allen and Garrett, 1971; Chen, 1985; Huxtable, 1986), synthesize Tau from cysteine,

though we have no information on which pathway(s) is(are) used in this conversion.

The incorporation of radioisotope into Ise by *Argiope* fed [^{14}C]Tau (Anderson and Tillinghast, 1980) needs verification, but if true would suggest that Ise is formed from Tau. Though this conversion apparently occurs in some organisms (Braun and Fromageot, 1962), it has not been established that Ise synthesis from Tau occurs in animal tissues (Huxtable, 1986; Fellman, 1987). More recent studies with human neutrophils have again raised the possibility of a Tau to Ise conversion in animals (Cunningham et al., 1998; Cunningham and Tipton, 2000), but the evidence is inconclusive.

Given the comparable specific activities obtained for Ise and the Tau moiety of NAT in the present study (Table 1), one possible alternative pathway to Ise (Cavallini et al., 1978; Scandurra et al., 1978) warrants mention. In this pathway, cystamine would give rise to equal amounts of cysteamine and 2-mercaptoethanol, from which the former can be converted to Tau (Cavallini et al., 1976; Huxtable and Bressler, 1976; Read

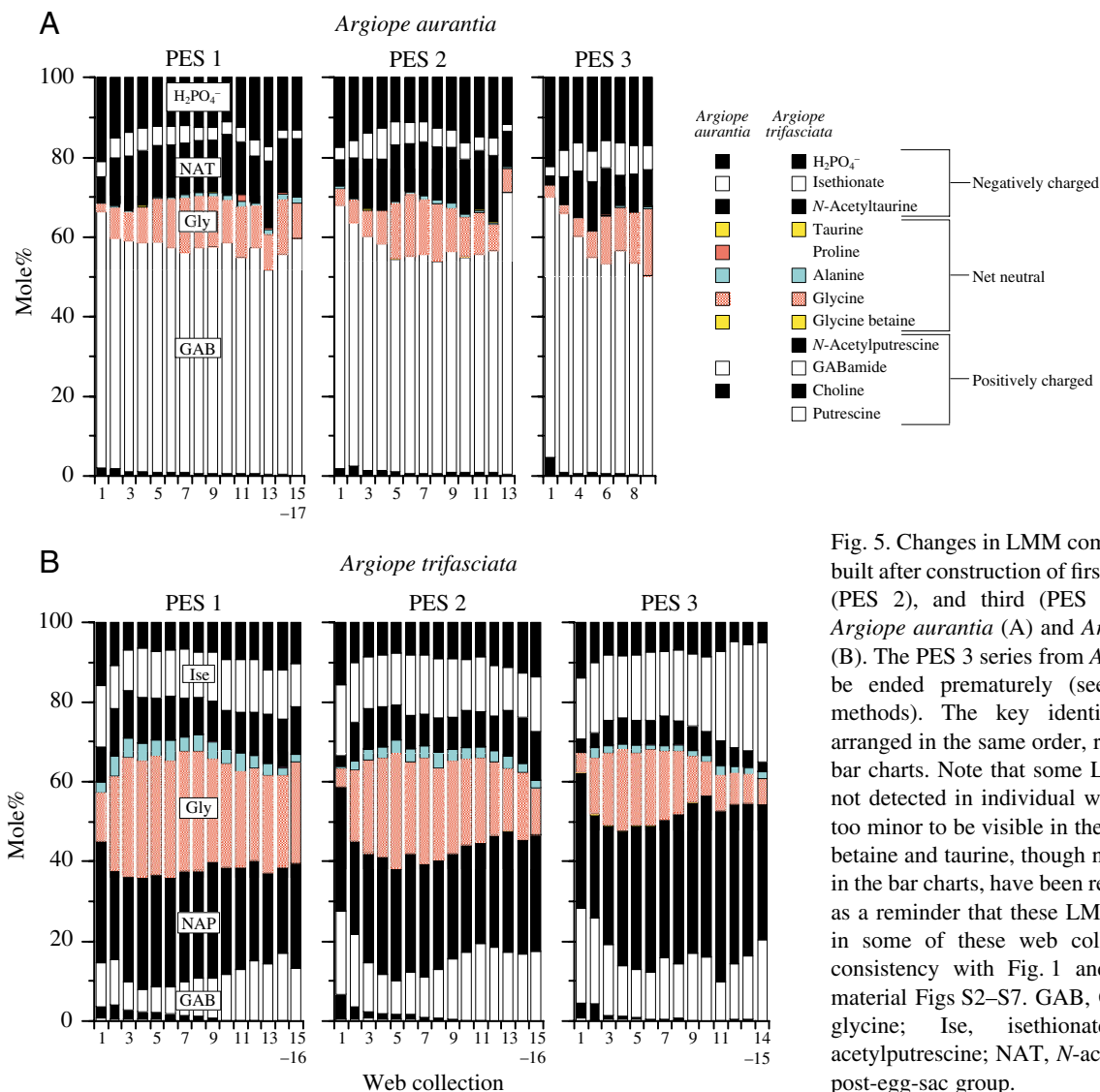


Fig. 5. Changes in LMM composition in webs built after construction of first (PES 1), second (PES 2), and third (PES 3) egg sacs by *Argiope aurantia* (A) and *Argiope trifasciata* (B). The PES 3 series from *A. aurantia* had to be ended prematurely (see Materials and methods). The key identifies the LMM, arranged in the same order, represented in the bar charts. Note that some LMM were either not detected in individual web collections or too minor to be visible in these plots. Glycine betaine and taurine, though not clearly visible in the bar charts, have been retained in the key as a reminder that these LMM were detected in some of these web collections and for consistency with Fig. 1 and supplementary material Figs S2–S7. GAB, GABamide; Gly, glycine; Ise, isethionate; NAP, N-acetylputrescine; NAT, N-acetyltaurine; PES, post-egg-sac group.

et al., 1976) and the latter to Ise (Federici et al., 1976; Dupré et al., 1978). Here, Tau is not a precursor, but a coproduct of the pathway to Ise.

NMR analyses have not revealed NAP in webs of *N. clavipes*, but Put is present in quantity (Higgins et al., 2001), as it is in some other araneoids [e.g. *Metepeira incrassata* F.O.P.-Cambridge 1903 (Higgins et al., 2001), *Micrathena gracilis* (Walckenaer 1805) (M. A. Townley and E. K. Tillinghast, unpublished)]. Put was radiolabeled in some webs built by *N. clavipes* fed [^{14}C]glucose or [^{14}C]acetate, but not as consistently as GAB, Gly, and Ala (Higgins and Rankin, 1999). Likewise, in this study the specific activity of the Put component of NAP was significantly lower than the specific activities of GAB, Gly and Ala (Table 1).

Choline and glycine betaine (poorly synthesized LMM)

In earlier studies, radioisotope was not observed in Cho from webs built by *A. aurantia* fed [^{14}C]glucose (Tillinghast and Townley, 1994) or *N. clavipes* fed [^{14}C]glucose or [^{14}C]acetate (Higgins and Rankin, 1999). Insects, likewise, are either unable to synthesize Cho or synthesize insufficient Cho (*via* methylation of phosphatidylethanolamine), making it a nutritional essential (Dadd, 1985). Limited synthesis of Cho from phosphatidylethanolamine also occurs in at least some crustaceans (Bilinski, 1962; Shieh, 1969; Brichon et al., 1980; D'Abramo and Baum, 1981), but again most evidence suggests that crustaceans have a dietary requirement for Cho (D'Abramo and Baum, 1981; Catacutan and de la Cruz, 1989; Kanazawa et al., unpublished; cited in Boonyaratpalin, 1996; Shiau, 1998; Shiau and Lo, 2001). Indeed, Cho's essential status may apply to arthropods generally (Morris, 1991).

In the present study, the specific radioactivity of Bet was not determined because of suspected contamination, but our observations indicate that labeling of this compound was slight at most. An earlier study found that Bet from webs of *N. clavipes* fed [^{14}C]glucose or [^{14}C]acetate was not radiolabeled and that this compound is likely essential (Higgins and Rankin, 1999). A significant positive correlation between molar percentages of Bet and Cho was reported in one population of *N. clavipes* (Higgins et al., 2001) and was likewise observed in all three species used in this study (Table 4). Such observations are consistent with Bet synthesis from Cho *via* betaine aldehyde, to our knowledge the only established pathway to Bet in both chelicerate (Dragolovich and Pierce, 1994) and mandibulate (Bilinski, 1960; Weiher and Komnick, 1997) arthropods.

Is there agreement between synthetic capacity and LMM compositional changes with starving?

Assuming an organic LMM's specific radioactivity is a reliable indicator of the spider's ability to synthesize that LMM, we anticipated that, with fasting, there would be decreased molar percentages of those LMM showing lower incorporation of radioisotope. Was this expectation met? In some respects, yes. Downward trends in starving spiders were most evident among 'poorly synthesized' (Cho, Bet) and

'moderately synthesized' (Ise, NAT, NAP, Put) LMM, with the exception of the decline in Ala in *A. trifasciata*. Extended upward trends were seen only among 'readily synthesized' LMM (GAB, Gly and, to a far lesser extent, Ala in *A. cavaticus*) and Pro (presumed at least a moderately synthesized LMM). Even more than increases in molar percentage, increases in $\mu\text{g web}^{-1}$ of Gly, GAB and Pro, as seen over at least part of a starving series in one or more species, suggest that starving spiders use these LMM to compensate to some extent for decreases in less readily synthesized and less available LMM.

Argiope trifasciata provided a particularly striking example of a compositional shift accompanying starvation that may have resulted from differences in synthetic capacity; specifically, the decline in molar percentage of NAP and coincident increase in GAB. In webs of this species, the mean specific radioactivity of GAB was almost four times higher than that of the Put moiety of NAP (Table 1). Unlike webs of *A. cavaticus* and *A. aurantia*, webs of *A. trifasciata* contain NAP as a major constituent whereas GAB is generally less abundant (Townley et al., 1991) and sometimes even a minor component, as at the start of the feeding and starving series (Figs 1, 2). This suggests that these two similar compounds may fulfill the same, as yet unknown, function in the sticky droplets and that when *A. trifasciata* are starved and their webs removed the more readily synthesized GAB is increasingly recruited to stand in for NAP. An inverse correlation between molar percentages of NAP and GAB in *A. trifasciata* (and *A. cavaticus*) is consistent with this interpretation (Table 4).

There were, on the other hand, a number of trends in the webs of starving spiders that did not conform to expectations based on specific radioactivities. Ala's unexpected decline in starving *A. trifasciata* has already been mentioned, but even the slight molar percentage increases in Ala in *A. cavaticus* and *A. aurantia* were scarcely commensurate with its specific radioactivity. Perhaps Ala is more in demand in starving spiders than Gly [e.g. as a substrate for gluconeogenesis (Felig, 1973)] and, thus, is less available for use in the web. We also did not anticipate the initial rise in Bet or NAT at the start of the series in starving *A. cavaticus*, when mass of the water-soluble fraction was already on the decline, nor the resurgence in Bet at the end of the series in both *Argiope* species. Also unexpected, Ise partially rebounded at the end of the starving *A. trifasciata* series, but the other major sulfonate, NAT, did not. Conversely, NAT partially rebounded at the end of the starving *A. aurantia* series, but Ise did not (Fig. 3). Neither rebounded in *A. cavaticus*. Probably not coincidentally, Ise is generally more abundant than NAT in webs of *A. trifasciata* while the opposite is true of *A. aurantia* webs (Vollrath et al., 1990; Townley et al., 1991). Given Cho's very low specific radioactivity, we were especially surprised that, after an initial decline, its molar percentage tended to level out higher than in the corresponding webs of the feeding groups. If the spiders' ability to synthesize Cho is so poor, where was it coming from in the starving spiders? One possibility is the store of Cho residing in membrane phospholipids, mobilized as tissue

reserves were broken down to meet the energy and material needs of vital tissues.

The most unexpected results, however, came from webs of the feeding controls. LMM composition was not as stable in these webs as we had anticipated. As detailed above, some compositional shifts in feeding group webs, especially of Gly, GAB, Pro, Cho, Bet, Ise and Put, were similar to trends observed in starving groups. Thus, some changes in composition with starvation are likely attributable, at least in part, to factors other than starvation shared by feeding group spiders. One such factor that almost certainly contributed to similar trends was the absence of web recycling, discussed in the following section. Another possibility, however, is that starving and feeding spiders were both responding to limited resources, but arising for different reasons; an absence of prey in starving groups *versus* allocation of resources to other activities, or the allocation of more resources to web building, in feeding groups. Examples of other activities include molting in juveniles and egg laying in adult females. These different possibilities are not mutually exclusive and the relative importance of each may differ among the different LMM.

For the present we are proceeding from the assumption that shifts in LMM composition seen in starving groups were shifts away from what would generally be a more effective composition for securing prey. This assumption may not be correct. At present we know almost nothing about how LMM compositional differences affect sticky spiral functioning.

Possible effects of web recycling on LMM composition

Spiders in the field often have the opportunity to recycle at least a portion of their old orb web by ingestion before they construct a new one (Hingston, 1922; Peakall, 1971; Carico, 1986 and references therein; Craig, 1989). In this study, however, spiders were only allowed to recycle their first post-ecdysial web; all other webs were collected for analysis. Web recycling or its lack clearly influences some web parameters (Breed et al., 1964) and this influence appears to extend to LMM composition. It seems likely that similar trends seen between feeding and starving groups were at least partly the result of these spiders being deprived of web material, particularly LMM, that they normally would have been able to recoup. It is especially likely that some differences seen between earlier and later web collections within a series were related to web recycling having occurred just before the start of the series, but not subsequently.

For example, we have evidence that the relatively steep decline in Cho early in the series in feeding and starving groups was the result of webs being recycled before, but not after, the start of the series. This evidence comes from an experiment in which we fed 4 male and 4 female penultimate instar *A. cavaticus* a solution containing 6.54×10^6 c.p.m. [$1,2\text{-}^{14}\text{C}$]Cho chloride (NEN, Boston, MA, USA) and 43.9 μg Cho. These spiders had all built two post-ecdysial webs, both of which we had removed, and had not been fed since prior to ecdysis. After radioisotope feeding, spiders received one fly (*P. sericata* or *P. regina*) after each of the first four webs built. We found that

$76.6 \pm 2.42\%$ (mean \pm s.e.m., $N=8$) of the ^{14}C ingested was present in the water-soluble fraction of the first 2 webs built; $81.5 \pm 2.59\%$ was present in the first five webs built. No significant difference was found between males and females comparing data from the first two webs ($P=0.646$) or webs 3–5 ($P=0.265$; unpaired *t*-test). Using the same protocol, we also attempted to feed four males and one female the same amount of [^{14}C]Cho to which an additional 224 μg unlabeled Cho was added, but only one male drank the solution quickly and without incident. The other spiders did eventually drink comparable, but imprecisely known, volumes. Nevertheless, with the one cooperative male, 75.0% of the ingested ^{14}C was present in the first two webs built; 78.3% was in the first six webs built. The results from the other four spiders, while only approximate, indicate that this result is representative.

Thus, it appears that a large percentage of ingested free Cho, such as the spider receives when it recycles an old web, is incorporated into future webs, with the bulk of this going into the first web. This high percentage of incorporation can occur even when relatively large quantities of free Cho (268 μg) are consumed, resulting in webs with unusually high molar percentages of Cho. For example, we analyzed the first labeled web built by the one female fed [^{14}C]Cho spiked with unlabeled Cho. Based on the 11 organic LMM constituting 100% of the LMM, Cho accounted for 60.6 mole% of the LMM in this web (!), much higher than we have ever seen in webs built by spiders fed only insects. By the second web, which contained only 13.8% as much ^{14}C as the first web, Cho had dropped to a more typical 11.8 mole%.

Considering the large contribution made by LMM to total web mass (see 'Gravimetric data' in Results) and the spider's limited capacity for synthesizing some of the LMM, the principal selective advantage in web recycling behavior may come from retrieval of LMM rather than silk protein residues (T. A. Blackledge, personal communication). As further suggested to us by Blackledge, this possibility is supported by observations indicating that those orb weavers that build webs lacking sticky droplets tend not to recycle their webs (e.g. the araneids *Cyrtophora* and *Mecynogea* (Lubin, 1986; Carico, 1986), and some, though apparently not all, uloborids (Eberhard, 1971; Opell, 1982; Lubin, 1986; Watanabe, 2001). However, the benefits from consuming water, very small insects (Nentwig, 1985) or pollen (Smith and Mommsen, 1984) when recycling webs cannot be discounted.

Possible effects of the molt/intermolt and egg laying cycles on LMM composition and web mass

We anticipated that the feeding group spiders might be less than ideal controls since they would be more apt to molt and lay eggs than starving group spiders. Indeed, most starving group juveniles did not molt and no starving group adults oviposited until feeding was resumed whereas all feeding group juveniles molted and some feeding group adults, though unmated, oviposited (supplementary material Fig. S1). If these factors affect the allocation of LMM to the web, then differences seen between feeding and starving groups may not

be attributable solely to changes resulting from starvation. For example, as noted earlier, the molar percentage of Cho tended to be higher in starving groups than in feeding groups as series progressed and in some of these later web collections even $\mu\text{g Cho web}^{-1}$ was higher in the starving groups. Was this difference due entirely to starvation, with free Cho liberated from membrane phospholipids as starving spiders tapped tissue reserves, or was it to some extent due to Cho in feeding spiders being diverted into reproduction or growth/molting?

Support for the second of these possibilities was provided by webs of PES group *Argiope* and juvenile *Araneus cavaticus*. In both *Argiope* species, Cho levels were highest in the first or second webs built after an egg sac was constructed. They dropped again with the approach of the next egg sac's construction (Fig. 5), suggesting that available Cho was being diverted away from foraging and into reproduction. In juvenile male and female *A. cavaticus*, $\mu\text{g Cho web}^{-1}$ were lower in the last web collections from feeding spiders than in the corresponding webs from starving spiders (supplementary material Table S4), suggesting that Cho was being diverted or held in reserve due to the impending molt.

The influence of reproduction and molting on LMM composition apparently extends beyond the above example with Cho. Cyclical changes in quantities of other LMM, synchronized to the egg laying cycle, are also evident in the data from the PES groups (Fig. 5). Certain end-of-series departures from earlier trends seen in feeding adults, but not feeding juveniles, such as declines in Gly, Ala, and Pro (Fig. 2), may also reflect the influence of egg laying. And in the latter half of the series from the *A. cavaticus* feeding juvenile male group (supplementary material Fig. S3), the drop in $\mu\text{g web}^{-1}$ of several LMM indicates that the reallocation of resources away from foraging applies to LMM in addition to Cho.

Growth/molting and reproduction may have contributed not only to differences between the webs of feeding and starving groups, but also to similarities. The reallocation of LMM due to growth/molting or reproduction in feeding groups may have produced shortages for web construction that in some ways resembled the effects of starvation. Perhaps some of the same changes in LMM composition made necessary by starvation are also favored in some circumstances by feeding spiders endeavoring to lay eggs or ecdyse. Reallocation of resources may be most evident in spiders subsisting on a diet that is quantitatively or qualitatively suboptimal, a topic we consider below.

The molt/intermolt and egg laying cycles also bring about changes in other web parameters, including, as seen in previous studies, web size, and as seen in this study, web mass. In a study with juvenile *Nephila clavipes* (where webs were not removed), orb web size typically increased in the earlier part of an intermolt and then decreased with the approach of the next ecdysis (Higgins, 1990). It has likewise been noted that *A. aurantia* build smaller webs around the time of molting (Reed et al., 1969). In our feeding juvenile *A. cavaticus* (where webs were removed), web mass did not change substantially

in the earlier part of the intermolt, but a clear decline in web mass with the approach of ecdysis was seen in males, though not females (supplementary material Figs S3, S4). Presumably, this difference between the sexes reflects a major difference following the final molt; males are unable to build orb webs (Sekiguchi, 1955) and thus much less likely to feed as adults than females. An initial increase in web mass in feeding adults, especially *Argiope* (Fig. 1, supplementary material Fig. S6), following the final molt may also reflect the influence of the molt/intermolt cycle. In *N. clavipes* (Higgins, 1990) and *Larinioides cornutus* (Clerck 1757) (Sherman, 1994), web size declined with the approach of egg laying. In this study, measurements of web mass indicated the same trend, seen most convincingly in the two *Argiope* species (the two species that produced egg sacs in the laboratory). Following oviposition, web size has been observed to increase in *L. cornutus* (Sherman, 1994) and we likewise observed web mass rebound in the two *Argiope* species after an egg sac was made (supplementary material Fig. S7).

The changes in web parameters apparently associated with the molt/intermolt and egg laying cycles may be considered in terms of resource allocation, with relative investments in foraging (the web primarily), growth and reproduction changing over time. But with molting and ovipositing, the influence of more tangible, anatomical changes should be considered as well. Several types of silk glands are remodeled during a molt, including those that produce the sticky spiral, the aggregate and flagelliform glands. At the height of remodeling these silk glands are nonfunctional (Townley et al., 1993) and orb web construction ceases for up to several days before ecdysis (e.g. Witt, 1971; Higgins, 1990). But beyond this clearcut effect on web building, it is possible that the last webs built before ecdysis and/or the first webs built after are the products of silk glands that are in the earliest or final phase of remodeling, respectively. If so, changes taking place in these silk glands might put constraints on certain web parameters such that some structural or compositional options, available at other times, are not available close to ecdysis.

There are also anatomical changes in the silk glands associated with egg development. It is not unusual for a large part of the abdomen to become increasingly crowded by eggs and cylindrical silk glands (major sources of egg sac silk) during this time. As a result, other tissues become compressed, including the aggregate glands, source of the web's sticky droplets. This compression may render these and other silk glands temporarily nonfunctional, forcing a suspension of web building until oviposition (Kovoor, 1972; Kovoor, 1977). Thus, comparable to silk gland remodeling during molting, the structure and composition of last webs built before oviposition may reflect silk glands experiencing reduced functionality.

Note that both molting and egg laying are often accompanied by a hiatus in web building extending before and/or after the day on which ecdysis or egg sac construction occur. This effect on the time between bouts of web building may itself influence LMM composition in the first post-ecdysial or post-ovipositional webs by affecting quantities of

LMM that can be synthesized or otherwise amassed for use in these webs.

Possible effects of feeding regimen on LMM composition among feeding group spiders

When setting the feeding regimen for the feeding groups it was our intention that spiders should not gain mass so fast that juveniles molted or adults oviposited after building only a few webs. There was a tendency for web mass to increase as the series progressed in feeding group spiders, particularly in the two *Argiope* species. How did the feeding regimen affect decisions regarding foraging investment? Did maintained or increased web mass resulting from such decisions contribute to compositional shifts similar in some respects to those seen in starving groups? We consider and provide some background to these questions in the following paragraphs.

Models (Sherman, 1994; Higgins, 1995) predict that foraging investment, represented principally by the web, increases as foraging success decreases, so long as the increased investment does not result in mass loss by the spider. At that point, further decreases in foraging success are accompanied by decreased foraging investment. Consequently, assuming average mesh size does not change, the largest webs are built by spiders consuming moderate amounts of food. Spiders consuming either very little or large amounts of food build smaller webs. Data from some studies on araneoid orb-weavers essentially agree with this model (Higgins and Buskirk, 1992; Sherman, 1994; Higgins, 1995; Herberstein et al., 1998; Herberstein et al., 2000; Tso, 1999; de Crespigny et al., 2001), as do studies on an uloborid orb-weaver (Watanabe, 2001). Other data are more ambiguous or in conflict with the model (Witt, 1963; Benforado and Kistler, 1973; Vollrath and Samu, 1997; Blackledge, 1998; Herberstein and Heiling, 1999), perhaps in part (1) because of constraints imposed on study animals (e.g. Blackledge, 1998), (2) because it is not well established what rates of consumption constitute low, moderate and high food diets, or (3) because a change in web size is not necessarily the only way a spider may manifest a change in foraging effort. An increase in foraging effort with decreased prey capture took the form of increased web size and web building frequency in a laboratory study with *A. trifasciata* (Tso, 1999), but only an increase in web building frequency in a laboratory study with *A. diadematus* (Vollrath and Samu, 1997).

Thus, was the tendency for web mass to increase in feeding group spiders, especially *Argiope*, an indication that the feeding rate was only moderate, a condition exacerbated by our removal of webs, and consequently foraging investment was increased? Or was the feeding rate more than moderate, resulting in a response (increased web mass) that would allow the spiders to better exploit the relatively abundant prey (Benforado and Kistler, 1973; Vollrath and Samu, 1997)? Or was increased web mass a response to a consistent, as opposed to a sporadic, return on the foraging investment (Herberstein et al., 2000)? There is also the possible influence of the molt/intermolt cycle on web mass, mentioned earlier.

Whichever, if any, of these four explanations apply, such increases in web mass may have spread certain resources too thin, such that compositional compromises were made by feeding group spiders. If resources were not only lost in the webs we collected, but were increasingly diverted into reproduction and/or growth by feeding group spiders, it would presumably have been even more difficult to maintain an optimal LMM composition while maintaining or increasing web mass. Thus, certain shifts in composition in feeding groups (e.g. the growing molar percentage of Gly) that were mirrored in starving groups may have reflected a compromise made by feeding spiders that enabled them to build heavier webs. Whether because of dietary deficiencies, synthetic capacity limitations, or diversion of resources into reproduction or growth, it may not always be possible to allocate the 'ideal' quantities of LMM to webs containing more aggregate gland secretion. Perhaps it is sometimes more advantageous to build heavier webs with a less-than-ideal LMM composition than to build lighter webs with the 'ideal' LMM composition.

Indeed, compositional compromises made under certain circumstances have previously been indicated in non-sticky web components. One study found that starvation may result in decreased extensibility of major ampullate silk (Madsen et al., 1999). They suggested that decreased availability of amino acids with starvation may result in compromised silks with compositions and mechanical properties different from those of well-nourished spiders. A related suggestion was made based on data indicating that qualitative differences in diet can influence the amino acid composition of major ampullate silk (Craig et al., 2000). The observation of considerable intraspecific and intraindividual variability in major ampullate silk amino acid composition lends support to these possibilities (Work and Young, 1987 and references therein; Craig et al., 2000), while the observation of a uniform intraspecific composition does not (Lombardi and Kaplan, 1990).

In comparing our study to earlier studies we should bear in mind differences in the web parameters measured. In this study, web mass was measured but dimensional parameters such as web size and mesh size were not, while in most earlier studies the converse is true. Ideally, the monitoring of foraging investment would include measures of both to help prevent or resolve seeming contradictions that can arise between studies. For example, in this study decreases in web mass were observed soon after starvation and web removal began, whereas in an earlier study no significant decrease in orb web size was observed after 6 days of starvation and web removal (Witt, 1963). And with resumed feeding of starved spiders there was no significant increase in web size even after 10 days of feeding (Witt, 1963), again in contrast to our mass results. However, Witt also obtained a measure of web mass in the form of total web nitrogen content which, divided by total thread length, was used to calculate 'thread thickness'. Witt notes that, despite web size remaining large after 6 days of starvation, there was a decrease in thread thickness in these webs, consistent with our mass data. Parenthetically, given the

large contribution the sticky spiral's aggregate gland cover makes to orb web mass and the recognition that many of the organic LMM contain nitrogen, we suggest that changes reported in 'thread thickness' with different feeding and/or web removal protocols (Witt, 1963; Breed et al., 1964) may be partly or largely attributable to changes in the quantity of aggregate gland secretion applied per length of sticky spiral. In our study, the tendency for the water-soluble percentage of the web to decrease over the series in many groups (supplementary material Table S3) indicates a decrease in this quantity. More direct observations have confirmed that this quantity varies, even within a single web (Eberhard, 1988; Vollrath and Edmonds, 1989; Edmonds and Vollrath, 1992).

In discussing the influence of the feeding regimen on compositional changes we should consider not only feeding rate but also quality of the diet. Of the three species used in this study, *A. cavaticus* is probably the most reliant on dipterans as a natural source of food (e.g. Olive, 1980; Riechert and Cady, 1983; Horton and Wise, 1983; Howell and Ellender, 1984). But even in this species a diet consisting of just one or a few species of brachyceran flies, as given to all feeding group spiders, is not typical of spiders in the field. Perhaps the same quantity of food, but from more varied or typical prey items, would have resulted in more stable LMM compositions in feeding group spiders. Qualitative aspects of the diet may also have contributed to differences in composition seen between radiolabeled and non-radiolabeled webs. As noted earlier, the molar percentage of Cho in the webs of *Argiope* given radioisotope was invariably higher than in the non-radioactive webs of conspecifics. The former had either recently been feeding in the field or were fed orthopterans, whereas the latter were fed only muscid and calliphorid flies. We note that a significant difference in the amino acid composition of *A. keyserlingi* dragline silk has been observed depending on whether the spiders had been feeding on blowflies or crickets (Craig et al., 2000).

Consistency amidst variation

While recognizing the significant intraspecific variation that clearly exists in LMM composition, and the validity of the statement made earlier (Higgins et al., 2001), that '*the composition of the organic low-molecular-weight solution is not fixed*', we should also recognize that there are features to a species' LMM composition that are at least typically maintained. The inventory of principal LMM is generally consistent within a species, at least within one sex, and some quantitative relationships among the LMM are often observed. In this study, GAB was invariably the most abundant LMM in webs of *A. aurantia* and *A. cavaticus* on a molar percentage basis (supplementary material Fig. S8). In *A. cavaticus* webs the molar percentage of NAT was almost invariably lower than that of Ise, often by a considerable margin (supplementary material Figs S3–S5), while the opposite was observed in *A. aurantia* (Fig. 5, supplementary material Fig. S6). Some quantitative relationships were observed in all three species. For example, Cho was usually (*A. trifasciata*) or always (*A.*

aurantia, *A. cavaticus*) more abundant than Bet, and Gly was usually (*A. cavaticus*) or always (*A. trifasciata*, *A. aurantia*) more abundant than Ala or Pro. Many other such relationships were demonstrated by ranking molar percentage data for each species (supplementary material Fig. S8).

When LMM carrying the same charge were grouped together, a consistency at a higher level was also evident within a species. Thus, the total molar percentage of positively charged organic LMM (GAB+NAP+Cho+Put) was generally maintained within a fairly narrow range even with starvation (mean \pm s.d. for: *A. cavaticus*, 54.4 \pm 3.08 mole%, $N=86$; *A. aurantia*, 59.0 \pm 4.07 mole%, $N=102$; *A. trifasciata*, 46.0 \pm 5.92 mole%, $N=123$) (Figs 1, 5, supplementary material Figs S2–S6). Relative to the means, standard deviations were larger for the total molar percentage of negatively charged organic LMM (Ise+NAT) (mean \pm s.d. for: *A. cavaticus*, 23.7 \pm 6.33 mole%; *A. aurantia*, 15.4 \pm 4.05 mole%; *A. trifasciata*, 24.8 \pm 4.94 mole%), apparently because of the low synthetic rates of Ise and the Tau portion of NAT. However, this greater variability was partially offset by compensatory changes in inorganic phosphate (mean \pm s.d. of Ise+NAT+H₂PO₄⁻ for: *A. cavaticus*, 38.8 \pm 5.05 mole%; *A. aurantia*, 31.2 \pm 4.41 mole%; *A. trifasciata*, 35.0 \pm 5.02 mole%).

Conclusions

We investigated how starvation affects LMM composition in orb web sticky droplets, anticipating that with fasting there would be decreases in the molar percentages of those organic LMM the spider is least able to synthesize and increases in those that are more readily synthesized. Many shifts in composition were basically consistent with differing synthetic capacities. Thus, declines in the molar percentages of LMM with lower rates of synthesis (Cho, Ise, NAT and, presumably, Bet) were observed at least over part of a web series in starving group spiders, while molar percentages of some LMM with higher rates of synthesis increased (Gly, GAB and, in *Araneus cavaticus*, Pro). The most convincing indications that certain more readily synthesized LMM were increasingly relied upon by starving spiders were increases observed in $\mu\text{g web}^{-1}$ of Gly, GAB (in *Argiope trifasciata*) and Pro (in *A. cavaticus*) in webs of starving group spiders. These increases in absolute quantities per web contrasted sharply with the decreases seen in most LMM in starving spiders and give the impression that these LMM were used to fill in for other, perhaps more desirable, but unavailable or costly LMM. In *A. trifasciata*, it appeared that GAB was increasingly used to substitute for the less readily synthesized NAP.

However, synthetic capacity was not an entirely reliable predictor of compositional changes with starvation. Some shifts in LMM molar percentages in starving group spiders were not predicted based on specific radioactivity measurements (e.g. the decrease in Ala in *A. trifasciata*). We also found a number of similarities between starving and feeding group spiders with respect to changes in LMM molar percentages, making interpretation of the results uncertain.

Possible explanations for these parallel changes include a factor common to both the feeding and starving groups. It is likely that one such factor was web recycling. By not allowing spiders to recycle their webs, a form of nutritional stress was imposed on feeding as well as starving group spiders and may have influenced LMM composition in a similar manner in both. It may also have contributed to decreases observed both in the water-soluble percentage of the web and the percentage of water-soluble mass that could be accounted for by the organic LMM. Paradoxically, it is also possible that the difference imposed on feeding and starving group spiders, availability of insect prey or lack thereof, contributed to similar molar percentage shifts. Feeding group spiders allocated resources to molting, egg laying and increased foraging (increased web mass), and these expenditures may have resulted in shortages of some LMM and consequent shifts in LMM composition, reminiscent of shifts due to fasting in starving group spiders. Analyses of webs of juveniles and adults indicated that both the molt/intermolt and egg laying cycles, respectively, influenced LMM composition.

Additional studies are needed to focus on individual factors that probably influence LMM composition, including those touched on in this report (web recycling, the molt/intermolt and egg laying cycles, qualitative and quantitative aspects of diet, time between web building episodes), as well as studies that focus on the synthesis, allocation, and transport of individual LMM.

List of abbreviations

Ala	alanine
Bet	glycine betaine
Cho	choline
2D-TLC	two-dimensional thin layer chromatography
GAB	4-aminobutyramide
Gly	glycine
¹ H-NMR	proton nuclear magnetic resonance spectroscopy
HVE	high-voltage paper electrophoresis
Ise	isethionic acid (2-hydroxyethane sulfonic acid)
LMM	sticky droplet low-molecular-mass compounds
NAP	N-monoacetylputrescine
NAT	N-acetyltaurine
PES	post-egg-sac
Pro	proline
Put	putrescine
Tau	taurine

This study would not have taken place without the aid of Kathy Gallagher (UNH Instrumentation Center), who went well above the call of duty in placing her expertise with NMR at our disposal, providing training and making large blocks of instrument time available to us. Nor would this study have been possible without partial financial support from the National Institutes of Health, grant R15DK/OD51291, to E.K.T. We are also very grateful to Dr Stacia Sower (UNH)

for the use of laboratory equipment and to Dr Todd Blackledge (University of Akron) and an anonymous reviewer for their many valuable suggestions for improving the manuscript.

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