Comparative spigot ontogeny across the Spider Tree of Life

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14 **Abstract:** Spiders are well known for their silk and its varying use across taxa. Very few studies

15 have examined the silk spigot ontogeny of the entire spinning field of a spider. Historically the

16 spider phylogeny was based on morphological data and behavioral data associated with silk.

17 Recent phylogenomics studies have shifted major paradigms in our understanding of silk use

18 evolution, reordering phylogenetic relationships that were once thought to be monophyletic.

19 Considering this, we explored spigot ontogeny in 22 species, including *Dolomedes tenebrosus*

20 and *Hogna carolinensis,* reported here for the first time. This is the first study of its kind and the

21 first to incorporate the Araneae Tree of Life. After rigorous testing for phylogenetic signal and

22 model fit, we performed 60 phylogenetic generalized least squares analyses on adult female and

23 second instar spigot morphology. Six analyses had significant correlation coefficients, suggesting

24 that instar, strategy, and spigot variety are good predictors of spigot number in spiders, after

25 correcting for bias of shared evolutionary history. We performed ancestral character estimation

26 of singular, fiber producing spigots on the posterior lateral spinneret whose potential homology

27 has long been debated. We found that the ancestral root of our phylogram of 22 species, with the

28 addition of five additional cribellate and ecribellate lineages, was more likely to have either none

29 or a modified spigot rather than a pseudoflagelliform gland spigot or a flagelliform spigot. This

30 spigot ontogeny approach is novel and we can build on our efforts from this study by growing the

31 dataset to include deeper taxon sampling and working towards the capability to incorporate full

32 ontogeny in the analysis.

33 **Introduction:**

34   Silk is the trait most commonly associated with spiders. Silk is produced by glands that service

35   spigots on specialized appendages called the spinnerets. Spinnerets are a distinguishable

36   synapomorphy of Araneae (Coddington, 1989; Platnick, 1990; Platnick et al., 1991; Griswold et

37   al., 2005; Wheeler et al., 2016). The morphology of the spinnerets and the silk spigots they

38   possess provides an advantage enabling spiders to create simple to complex silk structures from

39   sheet-webs to tangle webs (Selden et al., 2008). The evolutionary history of spiders has long

40   been explored in the context of silk evolution. With the arrival of molecular phylogenetics and

41   phylogenomics studies, our understanding of spider systematics has changed drastically from the

42   formerly well accepted hypotheses based on morphological and behavioral traits (Platnick, 1977;

43   Griswold et al., 2005; Bond et al., 2014; Fernández et al., 2014; Garrison et al., 2016; Wheeler et

44   al., 2016).

45   These recent updates have led to a paradigm shift in our perception of silk use evolution.

46   The most dramatic changes have occurred in the "Orbiculariae" where data mainly from orb web

47   weaving behavior provided weak corroborating evidence of monophyly, whereas contradictory

48   data were lacking. Specifically, the orb web was considered a key adaptation in spider evolution

49   (Bond & Opell, 1998). However, despite previous support for this hypothesis through

50   morphological and behavioral data (Coddington, 1986; Coddington, 1990; Hormiga & Griswold,

51   2014), the monophyly of Orbiculariae (cribellate Deinopoidea + viscous Araneoidea) has now

52   been rejected based on thorough molecular and phylogenomics studies (Dimitrov et al., 2012;

53   Bond et al., 2014; Fernández et al., 2014; Garrison et al., 2016; Wheeler et al., 2016). The former

54   "Orbiculariae" members Deinopoidea (cribellate orb builders) are now closer to the RTA clade

55   (includes wolf spiders and jumping spiders) rather than to the Araneoidea (sticky-silk orb

56   weavers) and Deinopoidea may not even be monophyletic (Garrison et al., 2016; Wheeler et al.,

57   2016).

58   Some studies of silk evolution have used web ontogeny as a tool to reconstruct ancestral

59   web conditions or plesiomorphic traits in silk use. Studies many from to the Araneoidea

60   (Robinson & Lubin, 1979; Eberhard, 1985; Eberhard, 1986; Barrantes & Madrigal-Brenes, 2008;

61   Barrantes & Eberhard, 2010), suggested that early instar webs and behavior resembled possible

62   ancestral states. In studies of both *Tengella perfuga* F. Dahl (1901) and *Tengella radiata* (W.

63   Kulczyński, 1909), early instar webs resembled simple sheet webs rather than the complex funnel

64   structures lined with cribellate silk observed in adults (Barrantes & Madrigal-Brenes, 2008;

65   Mallis & Miller, 2017). This may be the ancestral condition for this lineage. However, Mallis &

66   Miller (2017) observed *T. perfuga* lay down cribellate silk in an orb-like spiral within the

67   horizontal sheet. This observation makes sense, considering recent phylogenomics revisions and

68   results of the new Araneae Tree of Life (AToL) (Wheeler et al., 2016) project, where the sister

69   group to the RTA clade is now hypothesized to be the cribellate orb weavers of the Uloboridae

70   (Bond et al., 2014; Fernández et al., 2014; Garrison et al., 2016; Wheeler et al., 2016).

71   One of the current hypotheses about silk evolution is that there is an adaptive tradeoff

72   between fecundity and silk use that is driving spider evolution and where more recently derived

73   clades have lost silk as a foraging tool (Blackledge et al., 2009). Energy metabolism in a spider

74   species is related to natural history traits such as foraging activity level and courtship behaviors

75   (Anderson, 1970; Prestwich, 1977; Anderson & Prestwich, 1982; Prestwich, 1983; Anderson,

76   1996). Foraging activity level is also tied to the type of silk used and web building or non-web

77   building. Cribellate silk has been historically viewed as a plesiomorphic trait in spiders, which

78   requires the development and use of the cribellum (a plate derived from the ancestral anterior

79   median spinnerets) and the calamistrum (a setal comb on leg IV to pull the silk fibers out)

80   (Hawthorn & Opell, 2002; Blackledge et al., 2009; Pechmann et al., 2010). It is possible that that

81   trend toward higher fecundity that is observed in orb-weavers and non-web builders, when

82 compared to cribellate silk users supports the hypothesis of "adaptive escape" from metabolically

83 costly cribellate silk production and represents increased resource allocation to reproduction in

84 spiders that produce viscous silk or that are non-web building altogether (Blackledge et al.,

85 2009).

86   We wanted to explore the potential correlations of foraging strategy and silk use and did

87 so in the context of the silk spigots themselves. Each spigot is serviced by a specific gland and

88 each type of silk serves a different purpose. This has been most well explored in Araneidae

89 (Coddington, 1989; Yu & Coddington, 1990; Townley & Tillinghast, 2009; Garb, 2013). There

90 must be a caveat that in most cases the actual gland has not been observed and the spigot type is

91 assigned a gland type name based on inferential evidence such as position, fine structure and

92 ontogeny. Most of the Araneomorphae spiders possess five types of spigots (Griswold et al.

93 2005). These are the 1) major ampullate gland spigots (MAP) on the anterior lateral spinneret

94 (ALS), which produces dragline silk and structural silk for orb webs; 2) piriform gland spigots

95 (PI) on the ALS that produce silk that is used to attach the dragline to a substrate surface; 3)

96 minor ampullate gland spigots (mAP) on the posterior median spinneret (PMS), whose silk is

97 used as a temporary scaffolding for the spiral in the orb web and whose purpose in non-web

98 builders is not yet defined; 4) aciniform gland spigots (AC) on the PMS and 5) aciniform gland

99 spigots on the PLS, which produce silk used in prey wrapping and lining egg sacs, as well as the

100 sheet portions in non-orb webs. Another two types appear in the adult female instar of the

101 Entelegynae, i.e., spiders with sclerotized, flow-through female genitalia (Griswold & Ramírez,

102 2017) and their closest relatives (Austrochiloidea, Palpimanoidea and Leptonetidae), which

103 together form "CY spigot clade" (Wheeler et al., 2016). These are 6) cylindrical (=tubuliform)

104 gland spigots (CY) on the PMS and 7) cylindrical gland spigots on the PLS which are female

105 specific and produce fibers that form the egg sac (Fig.1, and see Fig. 1 in Garb, 2013). Because

106   they appear on two different and morphologically distinct spinnerets, we treat the AC and CY

107   gland spigots on the PMS and PLS separately. Araneoids also possess flagelliform (FL) gland

108   and aggregate (AG) gland spigots, which produce the sticky capture spiral in orb webs (Yu &

109   Coddington, 1990; Townley & Tillinghast, 2009; Garb, 2013). The former Deinopoids do not

110   possess flagelliform or aggregate gland spigots but instead possess a cribellum, paracribellar

111   spigots on the PMS (which attach the cribellate silk to its axial line), and the pseudoflagelliform

112   (PF) gland spigot (a possible MS spigot homologue) that produces the axial lines of cribellate

113   fibers (Hajer, 1991; Eberhard & Pereira, 1993). These cribellar fibrils serve as a prey-capture

114   mechanism, rather than the viscous capture spiral of orb webs. Other, more recently derived

115   cribellate spiders, such as the zoropsid *T. perfuga*, possess a modified spigot on the PLS which is

116   thought to produce the axial line (R.E. Alfaro, unpublished data). Zoropsids do not possess

117   paracribellar spigots on the PMS. However, in *T. perfuga*, the modified spigot is flanked by two

118   smaller, unknown spigots whose function is currently undetermined (R.E. Alfaro, unpublished

119   data; also observed in *T. radiata* in Griswold et al., 2005). We will refer to these flanking spigots

120   of the modified spigot as 'flankers'.

121   Adult spider silk spigot morphology has been used as a morphological character system in

122   many phylogenetic studies (Coddington, 1989; Platnick, 1990; Platnick et al., 1991; Griswold et

123   al., 2005; Ramírez, 2014). However, few studies have explored or incorporated the ontogeny of

124   the whole spinning field (Wąsowska, 1977; Yu & Coddington, 1990; Hajer, 1991; Townley &

125   Tillinghast, 2009; Dolejš et al., 2014; R. Carlson & C.E. Griswold, unpublished data). By

126   incorporating the ontogeny of the spinning field, we can observe when activity patterns shift in

127   one adult sex, as well as the appearance or disappearance of spigots, or whether certain spigots

128   increase in number with each molt to a new instar (Townley & Tillinghast, 2009; Mallis & Miller,

129   2017; R.E. Alfaro, unpublished data).

130   Here, we report for the first time the ontogeny of the spinning field of *Dolomedes*

131   *tenebrosus* N.M. Hentz (1884), a fishing spider (Pisauridae), and *Hogna carolinensis* (C.A.

132   Walckenaer, 1805), a wolf spider (Lycosidae). We also use the recently published AToL (Wheeler

133   et al., 2016) to conduct the first statistical phylogenetic comparative study of spigots and silk use

134   in spiders. We pooled these two datasets (*Dolomedes* and *Hogna*), as well as our previous study

135   of the cribellate zoropsid *Tengella perfuga*, along with five previously published studies and one

136   unpublished dataset (Wąsowska, 1977; Yu & Coddington, 1990; Hajer, 1991; Townley &

137   Tillinghast, 2009; Dolejš et al., 2014; R.E. Alfaro, unpublished data; R. Carlson & C.E. Griswold,

138   unpublished data).

139   The main objective of our study is to explore trends in silk evolution using phylogenetic

140   comparative methods focusing on correlations between predictor variables such as foraging

141   strategy, and response variables such as the average number of aciniform spigots on the PMS.

142   Therefore, considering the spigot ontogeny of several species across the phylogeny with various

143   foraging strategies and types of silk expressed, and the new Araneae Tree of Life (Wheeler et al.,

144   2016), we may be able to disentangle the variables that are correlated such as spigot number,

145   type, foraging strategy and determine what may be driving silk use evolution after correcting for

146   shared evolutionary history (phylogenetic correction). The four questions guiding our approach

147   are: 1) Is there a relationship between foraging strategy (web vs. non-web) or specific foraging

148   strategies (i.e. ambush, active, sit & wait, etc.) and the number of certain silk spigots in spiders?

149   2) Is the overall diversity of spigots possessed by a species correlated with spigot number? 3)

150   Does ontogeny have an effect on the number of spigots? and 4) Are there homologous spigots

151   across taxa, particularly the singular, fiber producing spigot (MS, FL, PF) the on the PLS?

152   **Materials & Methods:**

Spider husbandry:

Rearing conditions and lab colony maintenance for *Hogna carolinensis* and *Dolomedes*
tenebrosus follow Mallis & Miller (2017). The founding female *H. carolinensis* was collected in
Bernalillo County, New Mexico (D. Lightfoot, 10-Sept-2014) carrying second instar spiderlings
on her abdomen. A gravid *D. tenebrosus* with an egg sac was sent to us from Bedford County,
Virginia (K. Benson, 29-June-2014). When possible, two to three replicates of each instar were
randomly sacrificed for scanning electron microscopy (SEM) imaging to account for variation
among individuals of the same instar. After developing through the first few instars of both
species, colony survival strongly declined. Subsequently, single samples were collected at each
instar for *D. tenebrosus*, while after the seventh instar in *H. carolinensis*, a single female was
followed to adulthood (twelfth instar). The founding females, as well as instar vouchers, were
deposited at the Museum of Southwestern Biology, Division of Arthropods (MSBA 50049 –
50070).

SEM preparation, imaging and spigot mapping:

We dissected in 100% EtOH, and then critical point dried using a Denton DCP-1 critical point
dryer, and mounted specimens of each instar for *D. tenebrosus* and *H. carolinensis* onto standard
SEM stubs using a combination of copper tape and non-conductive glue at California Academy
of Sciences (CAS). Finally, all SEM stubs were coated in gold/palladium using a Cressington
Sputter coater 108 (6002, 6006 series) that used Argon gas to facilitate coating. At CAS, we
obtained SEM images on the Hitachi SU-3500 scanning electron microscope. Up to 20 views of
each instar spinning field for both species were captured, covering all spinnerets for two
replicates of each instar. These SEMs were used to create spigot maps which were translated into
a spigot ontogeny dataset for each species (methods outlined in Coddington (1989) and Griswold
et al., (2005); see Table 1). Spigot mapping allows for notation of type, functionality and

177   placement of silk spigots on each spinneret, as well as tracking the growth of the spinning fields

178   from instar to instar and the appearance of interesting spigot formations.

179   **Spigot ontogeny datasets:**

180   We compiled a large spigot ontogeny dataset of 22 species comprising thirteen spider families

181   using previously published studies and unpublished datasets (Table 2; Wąsowska, 1977; Yu &

182   Coddington, 1990; Hajer, 1991; Townley & Tillinghast, 2009; Dolejš et al., 2014; R. Carlson &

183   C.E. Griswold, unpublished data). Three of those sources came from our lab colonies not only

184   for *D. tenebrosus* and *H. carolinensis*, as reported here but also the cribellate zoropsid, *Tengella*

185   *perfuga* (R.E. Alfaro, unpublished data). The dataset included the appearance, type, and number

186   of specific spigots on each spinneret (Fig. 1, Table S1). While some studies reported tartipores

187   (remnant structures of spigots from previous instar spigots) and others the presence of nubbins

188   (non-functioning spigots), all studies reported the number of the seven common (shared) spigot

189   types across all the species of this study: 1. MAP gland spigots, 2. PI gland spigots, 3. mAP

190   gland spigots, 4. AC gland spigots on the PMS, 5. CY gland spigots on the PMS, 6. AC gland

191   spigots on the PLS and 7. CY gland spigots on the PLS. We standardized the final data set to

192   include these 'standard 7' spigots (Table S1).

193   **Variables of Interest:**

194   From the standardized datasets, we selected five independent or predictor variables for

195   phylogenetic comparative analyses, and chose twelve dependent or response variables for the

196   final analyses. The independent variables were (Table 2):

197   1. Strategy: Foraging strategy scored as 0: web builder or 1: non-web builder.
198   2. Specific: Specific foraging strategies, scored as 1: sit & wait, 1.5: ambush, 2: sit &

199   pursue, 2.5: stalking, 3: active, 4: sheet web, 4.5: funnel web or 5: orb web. Sit & pursue,

200   while similar to sit & wait, is defined as an intermediate state between sit & wait and

201   active hunting (Schmitz & Suttle, 2001, Miller et al., 2013). With a sit & wait strategy,

202   the otherwise motionless spider grabs the prey when it comes within striking distance,

203   whereas with sit & pursue, the otherwise motionless spider runs after the prey when it

204   comes within several centimeters proximity and pursues the prey until captured (Schmitz

205   & Suttle, 2001, Miller et al., 2013).

206   3. Silk: Main type of silk used, scored as 1: none/MAP dragline, 1.5: burrow, 2: aciniform

207   sheet, 3: cribellate, or 4: viscous silk, i.e., that produced from the aggregate gland spigots.

208   4. Type: A measure of the variety of spigots the species possessed beyond what we called the

209   standard 7. These scored as 1: standard 7, 1.5: standard 7 + modified piriform gland

210   spigots on the ALS, 2: standard 7 + modified spigot on PLS, 2.5: standard 7 + modified

211   spigot on PLS with two 'flankers' + cribellum, 3: standard 7 + aggregate and flagelliform

212   gland spigots on the PLS or 4: standard 7 + cribellum, paracribellar gland spigots on the

213   PMS and a pseudoflagelliform gland spigot on the PLS.

214   5. Instar: Maximum number of instars that a species goes through to reach adulthood

215   (females).

216   The twelve dependent variables were continuous and were the average number of spigots for

217   each of the 'standard 7' found in all the spider species spinning fields. We focused on the adult

218   female instar as well as the second instar, i.e., when all spiders emerge from the egg sac.

219   Specifically, for adult females, the seven dependent variables were average number of spigots for:

220   1. MAP gland spigots on the ALS, 2. PI gland spigots on the ALS, 3. mAP gland spigots on the

221   PMS, 4. AC gland spigots on the PMS, 5. CY gland spigots on the PMS, 6. AC gland spigots on

222   the PLS and 7. CY gland spigots on the PLS. For second instars, the five dependent variables

223   were the average number of spigots for: 8. MAP gland spigots on the ALS, 9. PI gland spigots on

224   the ALS, 10. mAP gland spigots on the PMS, 11. AC gland spigots on the PMS and 12. AC gland

225   spigots on the PLS. Cylindrical gland spigots are only found in adult female spiders, which is

226   why they are not included with the second instars.

227   Phylogenetic comparative analyses have typically dealt with at least two continuous

228   variables. Methods have improved to accommodate the increase in Type 1 Error associated with

229   discrete variables such that analyses like phylogenetic generalized least square models (PGLS)

230   can be robustly performed (Graber, 2013; Maddison & FitzJohn, 2015). While theoretically

231   repeated-measures or a factorial ANOVA are possible in a phylogenetic context, the methods of

232   incorporating them into phylogenetic comparative analyses are not yet developed (Guo et al.,

233   2007). Thus, our time series study could not be analyzed as a whole unit. To gain a picture of the

234   potential effects of ontogeny, we performed several PGLS analyses on the adult female spigot

235   numbers as well as those of the second instars. These include the stage at which all spiders

236   emerge from the egg sac and are the most similar in condition (second instars) to the stage when

237   the most diversification and growth in spigots appears (adult female stage).

238   **Phylogeny:**

239   We used the topology of the recently published Araneae Tree of Life (AToL) (Wheeler et al.,

240   2016) for the 22 species included in our study. At the time of publication of this manuscript, the

241   AToL sequences have not been publicly released. Thus, while we were not able to generate a

242   time-calibrated tree or one with branch lengths equal to a rate of molecular evolution, we could

243   use the published topology and create two phylograms. One phylogram had all branch lengths

244   equal to 1 and the other was an ultrametric tree with the same topology (Fig. 2), using the *ape*

245   (version 4.1) package in *R* (Paradis et al., 2004). Both trees were used in model testing for

246   phylogenetic comparative analyses and ultimately, we used the ultrametric topology in our final

247   analyses.

248   **Phylogenetic comparative analyses:**

249   All analyses were performed in *R* using *RStudio* (version 3.2, R Core Team, 2016; RStudio,

250   2017). First, we tested both sets of dependent and independent variables for phylogenetic signal

251   using the *phytools* (version 0.5-64) package in *R* (Revell, 2012). All independent variables had

252   strong phylogenetic signal with *Blomberg's K* (Blomberg et al., 2003) and *Pagel's λ* (Pagel, 1999)

253   being close to 1 and with significant p-values while most of the dependent variables did not show

254   phylogenetic signal (Table 3). Despite this, given the strong signal in the predictor variables, we

255   decided to proceed with PGLS analyses. Because the K values were so close to 1, a Brownian

256   motion model of evolution was the best fit. We further tested various models of evolutionary

257   rate, and confirmed the Brownian model of evolution being the best fit (Results S1). Next, using

258   both trees, we tested PGLS models using generalized least squares method of model selection

259   with single term up to all five independent variables included. We used the *R* packages, *nlme* and

260   *MuMIn* (version 1.15.6) to test model fit (Bartón, 2016; Pinheiro et al., 2017). PGLS analyses

261   required the use of the *ape*, *geiger* (version 2.0.6), *nlme* (version 3.1-131) and *phytools* packages

262   in *R* (Paradis et al., 2004; Harmon et al., 2008; Revell, 2012; Pinheiro et al., 2017). With delta

263   AICc values equal to zero or very close to zero between terms, and a significant p-value

264   associated with the single term Instar, we determined that single term models were the best fit

265   with the trees and the datasets for PGLS as there was no significant effect of adding additional

266   terms (Table 4, Results S2). We ran a total of 35 PGLS analyses covering each of the adult

267   female dependent variables (average numbers of specific silk spigots) and 25 PGLS analyses

268   covering each of the second instar dependent variables using the *ape* and *geiger* packages in *R*

269   (Paradis et al., 2004; Harmon et al., 2008). We did this with both trees, and while the specific

270   analyses results differed, the main conclusions did not. We also performed ANOVA analyses on

271   the independent variable means derived through the PGLS models. Because all the independent

272   variables were discrete the ANOVA analyses served to corroborate the PGLS results. Here we

273   report the significant PGLS coefficient of correlation results (Table 5) using a maximum

274   likelihood approach, with a Brownian model of evolution and the ultrametric tree. The 60 full

275   PGLS analyses and ANOVA results using the ultrametric tree are available in Results S3, S4.

276   **Ancestral character estimation**:

277   Finally, we used the ultrametric tree and the *ace* function in the *ape* package in *R* (Paradis et al.,

278   2004) to conduct ancestral character estimation on the diverse, singular spigots found on the PLS

279   to explore the unresolved question of whether these spigots are homologous structures or not.

280   The spigots of interest were the modified spigot (MS) found in some cribellate and ecribellate

281   spiders, the pseudoflagelliform gland spigot (PF) found in cribellate orb weavers and the

282   flagelliform gland spigot (FL) found in viscous orb weavers. We added five additional taxa,

283   using data derived from adult female SEM images in Griswold et al. (2005) to allow for broader

284   taxon sampling deeper into the phylogeny. These species were the cribellate *Hypochilus pococki*

285   N.I. Platnick (1987; Hypochilidae), *Kukulcania hibernalis* (N.M. Hentz, 1842; Filistatidae),

286   *Thaida peculiaris* F. Karsch (1880; Austrochilidae), *Megadictyna thilenii* F. Dahl (1906;

287   Megadictynidae) and ecribellate *Nicodamus mainae* M.S. Harvey (1995; Nicodamidae). The

288   Nicodamoidea (Megadictynidae plus Nicodamidae) are sister to all Araneoidea, while the other

289   families are sister to the Araneoidea + RTA clades (Wheeler et al., 2016). We used a maximum

290   likelihood method with a model of the weighted rate matrix of substitutions for these spigots

291   (Table 6). As we describe below, we constrained our rate matrix (Table 6) that we used to model

292   substitution rates across branch lengths based on prior knowledge about historical possession of

293   the flagelliform gland, pseudoflagelliform gland and modified spigots in each taxon (Table 6)

294   (Platnick et al., 1991; Griswold et al., 2005; Dimitrov et al., 2016; Wheeler et al., 2016). Some of

295   the more recently derived clades within the Araneoids (Dimitrov et al., 2016) have lost

296   flagelliform gland spigots (i.e. Mimetidae, Arkyidae). We ranked the transition to Loss (of

297   spigots) as 1 substitution and that to none, pseudoflagelliform, or modified spigots (not observed

298 anywhere in this lineage) as 0 substitutions, or no likelihood (Table 6). Because they are found in

299 cribellate groups ancestral to both the RTA and Araneoid clades, we ranked all transitions for

300 modified spigots to the other four states as 1 substitution (Table 6). One interpretation of Dollo's

301 Law is that it is easier to lose a structure than to re-evolve it (Dollo, 1893) and this influenced

302 how we weighted the remaining matrix for Pseudoflagelliform, Loss, and the possible None (no

303 spigot) cases. Since one of the cribellate ancestral species, *Kukulcania hibernalis,* does not

304 possess anything corresponding to a modified or pseudoflagelliform gland spigot, and is

305 classified as 'None', we conservatively allowed for a single substitution from None to

306 Flagelliform, Modified and Pseudoflagelliform in our rate matrix (Table 6). Finally, since the

307 cribellate orb weaving sister group to the RTA clade possesses a pseudoflagelliform gland spigot

308 (PF) and RTA clade members possess modified (MS) or no spigots, we allowed for 0

309 substitutions in this direction. We also allowed for a substitution rate of 1 between

310 Pseudoflagelliform and Flagelliform as we do not know whether the ancestral orb weaver

311 possessed pseudoflagelliform or modified spigots (Bond et al., 2014; Garrison et al., 2016;

312 Wheeler et al., 2016). We then plotted the likelihoods of states at each node on the ultrametric

313 phylogeny (Fig 3, Results S5).

314   Additionally, we performed ancestral character estimation analyses for four of the five

315 independent variables from the PGLS analyses on the ultrametric tree with increased taxa:

316 Strategy, Specific, Silk and Type (Results S5). The constrained rate matrices for each variable

317 along with explanations for the given matrices are provided in Table S2. We did not include

318 maximum instar, as those data were not available in the literature for the five additional taxa.

319 Also, due to its variability within a species (see Table S1), ancestral character estimation may not

320 be a useful tool to use to understand maximum instar in silk evolution.

321   **Results:**

322   **Spigot ontogeny of *Dolomedes tenebrosus* and *Hogna carolinensis*:**

323   All instars of *D. tenebrosus* (instars 2-13) and more than half of the instars (instars 2-7 and 12) of

324   *H. carolinensis* were observed and sampled from lab colonies for SEM imaging to assess the

325   spigot ontogeny of the full spinning field of these lycosoid spiders (Homann, 1971; Griswold,

326   1993; Polotow et al., 2015). *Dolomedes tenebrosus* reached adulthood in thirteen instars while

327   *H. carolinensis* reached adulthood in twelve (Table 1).

328   *Anterior lateral spinnerets*: Both species possessed two MAP gland spigots, except for the adult

329   male stage (Table 1, Fig. 4). Piriform gland spigots increased in number to adulthood (Fig. 4).

330   However, in adult male *D. tenebrosus,* PI gland spigots decreased from the penultimate instar,

331   which led to sexual dimorphism (Table 1). It was not until later instars of *D. tenebrosus* and *H.*

332   *carolinensis* that the number of PI gland spigots increased in greater magnitude from instar to

333   instar (Figs. 4A-E, Table 1). We also observed sensilla (sensory pores) in the MAP fields of both

334   species (Fig. 5).

335   *Posterior median spinnerets*: Both species possessed two mAP gland spigots, except for the adult

336   male stage of *D. tenebrosus* (Table 1, Fig. 6). CY gland spigots did not appear until the adult

337   female and probably penultimate female instars. Penultimate *D. tenebrosus* possessed at least

338   one pre-cylindrical gland spigot (Fig. 6F). *D. tenebrosus* adult females bore many more CY

339   gland spigots than *H. carolinensis* (Fig. 6C). In *D. tenebrosus*, AC gland spigot numbers did not

340   change at 4 AC gland spigots for instars 2/3, then at 5 AC gland spigots through instars 4/5/6, and

341   then 8 AC gland spigots for instars 8/9/10 (Table 1, Figs. 6D, E). Both males and females lost

342   AC gland spigots in the final molt to adulthood. In *H. carolinensis*, AC gland spigots dropped in

343   number at instar 4 and remained at 3 AC gland spigots for the next two instars (Table 1, Figs. 6A,

344   B). By the adult instar, the number was far greater in the female *H. carolinensis* than the female

345   *D. tenebrosus* (Table 1, Fig. 6C).

346   *Posterior lateral spinnerets*: Cylindrical gland spigots also appeared in the penultimate female

347   stage with the three pre-spigots visible (Fig. 7F). Adult female *H. carolinensis* possessed a single

348   CY gland spigot compared to the 28 CY gland spigots in adult female *D. tenebrosus* (Table 1).

349   Aciniform gland spigots presented different trends between the two species (Table 1, Figs. 7, 8).

350   In *D. tenebrosus*, AC gland spigot numbers slowly increased with the same number persisting for

351   2-3 instars, then increasing (Fig. 7). We also observed loss of spigots in the final molts to

352   adulthood in both males and females (Table 1). In *H. carolinensis*, a sharp decrease to 3 AC

353   gland spigots occurred in instar 4 and persisted through instar 5, then increased to 7 AC gland

354   spigots in instar 6 persisting through instar 7 (Table 1, Fig. 8). The female *H. carolinensis*

355   possessed more AC gland spigots than the female *D. tenebrosus*. In both species, a larger spigot

356   was tentatively identified as a 'modified spigot' (see Griswold et al., 2005: 61; character 96), with

357   a potential pre-modified spigot observed in the penultimate female stage of *D. tenebrosus*. These

358   made no other appearance in the ontogeny the spinning fields of either species (Table 1, Figs. 7,

359   8).

360   **Phylogenetic comparative analyses:**

361   We compiled a full spigot ontogeny dataset of 22 species representing thirteen spider families and

362   scored the five independent, predictor variables: Strategy, Specific, Silk, Type, Instar (Table S1,

363   Table 2). After determining that the independent variables had strong phylogenetic signal,

364   whereas only three of the twelve dependent response variables did, we decided to proceed with

365   the phylogenetic generalized least squares analyses (Table 3). After thorough tree and model

366   selection analyses, we determined that single term models were the best fit along with the

367   ultrametric tree and a Brownian model of evolution (Table 4, Fig. 2). We ran a total of 60 PGLS

368   analyses (Results S3, S4) and here report the significant results of those analyses (Table 5).

369   In the adult female analyses, we found that Instar was a significant predictor in a few

370   cases. For piriform gland spigots (ALS), the coefficient of correlation between Instar and

371   average number of spigots was significant (Table 5). This was also the case for aciniform gland

372   spigots of the PMS (Table 5). For female PI gland spigots, Type, or the variety of spigots

373   possessed, was also a significant predictor of Average number of PI (Table 5). Of interest, the

374   female mAP gland (PMS) spigots did show strong phylogenetic signal and Strategy was a

375   significant predictor of Average number of mAP gland spigots. This means that, after the bias of

376   the correlation due to phylogeny was accounted for through the PGLS, the species classification

377   of either being a web builder or not had a strong coefficient of correlation with the average

378   number of mAP gland spigots possessed (Table 5).

379   In the second instar analyses, we found that Instar was also a significant predictor or

380   independent variable. For MAP gland (ALS) spigots in second instars, the coefficient of

381   correlation between Instar and Average number of MAP gland spigots was significant (Table 5).

382   Spiders possessed one, two or no MAP gland spigots at the emergence from the egg sac (second

383   instar) (Table S1). For the mAP gland (PMS) spigots, which did show strong phylogenetic signal

384   in preliminary testing, Instar was a significant predictor of the Average number of mAP gland

385   spigots (Table 5). Second instar spiders possessed two, one or no mAP gland spigots at this first

386   stage outside of the egg sac (Table S1).

387   **Ancestral character estimation of singular spigots on the PLS:**

388   We performed a maximum likelihood ancestral character estimation (ACE) of potentially

389   homologous spigots producing axial lines, i.e., the singular spigot on the PLS. We used the

390 ultrametric tree and overall scoring of whether a species possesses a spigot and if so, which

391 spigot it is: Flagelliform, Modified, None (never historically possessing a singular spigot),

392 Pseudoflagelliform, or Loss (previously possessing a singular spigot) of a spigot. We used a

393 constrained rate matrix for the five states as our model of substitution rates for the ACE analyses

394 (Table 6). The log likelihood value for the analysis was -46.023. Within this analysis we

395 calculated the scaled likelihoods of states at the root (Flagelliform gland spigot: 0.000, Modified

396 spigot: 0.365, None (no singular spigot that produces axial lines): 0.635, Pseudoflagelliform

397 gland spigot: 0.000, Loss: 0.000), as well as the other nodes of the phylogram produced (Fig. 3,

398 Results S5). In our color-coded results, the predominately red clade was the Araneoidea, which

399 includes viscous orb weavers (FL spigot) and the pirate spiders (Mimetidae) which do not possess

400 a FL spigot as adults (Loss) (Fig. 3). However, as very young juveniles mimetids possessed

401 vestigial FL and aggregate gland spigots on the PLS (Table S1, Townley & Tillinghast, 2009).

402 The predominately green clade (MS spigot) was the RTA clade, with loss of MS spigot (black) in

403 many of the tip species, while just sister to that were the yellow clades (Pseudoflagelliform),

404 which included the cribellate sheet (*Phyxelida*) and cribellate orb weavers (*Hyptiotes*).

405 Additional plesiomorphic cribellate taxa, possessing MS spigots (green, *Thaida, Hypochilus*) or

406 no unique spigot on the PLS (blue, *Kukulkania*) were included for deeper phylogenetic sampling

407 (Fig. 3). Finally, the results suggested that, at the ancestral root node, the PLS singular spigot

408 was more likely to have been a modified spigot than a pseudoflagelliform gland spigot, and that

409 the ancestor either possessed a modified spigot or no singular spigot (Fig. 3, Table 4).

410 We followed a similar procedure for maximum likelihood ancestral character state

411 estimation for four of the five independent variables: Strategy, Specific, Silk and Type. The

412 constrained rate matrices used as the model for the analyses are given in Table S2. The resulting

413 state likelihood values for each node are also given in Results S5 with the previous ACE node

414   results. For the variable Strategy (foraging strategy), the ancestral character estimation model has

415   a log likelihood of -14.73. At the root, the ancestral state was "Web" or web building (likelihood

416   = 1.0) rather than no web or loss of web (Fig 9, Results S5). Therefore, the ancestral state for the

417   araneoids and lycosoids was a web builder. Specific (specific foraging strategy) ACE model has

418   a log likelihood of -58.65. At the ancestral node, the states are for web building (Fig 10). The

419   ancestor of lycosoids and araneoids likely spun a funnel web (likelihood = 0.812) or a sheet web

420   (likelihood = 0.084); an orb web (likelihood = 0.105) is less likely (Fig 10, Results S5). The

421   ACE model for Silk (main type of silk expressed) has a log likelihood of -44.61. The ancestor of

422   araneoids and lycosoids produced cribellate silk (likelihood = 1.0; Fig 11, Results S5). Finally,

423   the ACE model for Type (variety of silk spigot types) had a log likelihood of -36.14. The

424   ancestral state at the root node was undetermined, but the two states with the highest likelihoods

425   were cribellate spiders possessing either Standard 7 + Cribellum + Paracribellar spigots

426   (likelihood = 0.398) or Standard 7 + Cribellum + Pseudoflagelliform + Paracribellar spigots

427   (likelihood = 0.433). All additional five states had much lower likelihoods (Fig 12, Results S5).

428   **Discussion:**

429   This is the first published full ontogeny of the spinning apparatus of both *D. tenebrosus* and *H.*

430   *carolinensis*. This is also the first statistical phylogenetic comparative analysis exploring

431   questions in silk use and evolution across several spider taxa. By creating a standardized dataset

432   across 22 species, we could unite the few existing spigot ontogeny studies into a comparative and

433   phylogenetic context (Wąsowska, 1977; Yu & Coddington, 1990; Hajer, 1991; Townley &

434   Tillinghast, 2009; Dolejš et al., 2014; R. Carlson & C.E. Griswold, unpublished data; R.E. Alfaro,

435   unpublished data).

436 **Spigot ontogeny of *D. tenebrosus* and *H. carolinensis*:**

437 Both *D. tenebrosus* and *H. carolinensis* are large-bodied lycosoids. *Dolomedes tenebrosus*

438 belongs to the Pisauridae family, the fishing or nursery web spiders, and employs a sit & wait

439 foraging strategy (Table 2) whereas *H. carolinensis*, belonging to the diverse Lycosidae family,

440 employs a sit & pursue strategy (Table 2) (Schmitz & Suttle, 2001, Miller et al., 2013). While

441 both have a similar number of instars to adulthood, the two species differ dramatically from each

442 other in the loss and regain of aciniform gland spigots on the PMS and PLS (Table 1, Figs. 6, 7,

443 8). In *D. tenebrosus*, AC gland spigot numbers on the PMS do not change for multiple instars

444 slowly increasing after two to three instars with a dramatic increase in the final molts. In *H.*

445 *carolinensis,* AC gland spigot numbers drop and remain low for two to three instars before

446 gradually increasing, until a similar dramatic increase in number with the final molt (Table 1, Fig.

447 6). The same interesting trend was also observed for aciniform gland spigots on the PLS in both

448 species (Table 1, Figs. 7, 8). These trends were not observed in other lycosoids previously

449 studied including other members of the Lycosidae (Wąsowska, 1977; Dolejš et al., 2014). *H.*

450 *carolinensis* do not form webs. *D. tenebrosus* instars were observed in the lab to build silk

451 scaffolding where they rested and at times fed (R.E. Alfaro, unpublished data). It is possible that

452 this difference in silk use and foraging strategy between *H. carolinensis* and *D. tenebrosus*,

453 especially the lack of regular web building, could account for the trends we observed with AC

454 gland spigot numbers. Both species were observed on a few occasions to wrap prey items after a

455 preliminary bite before returning to bite again. This is an ancient behavior seen in many other

456 taxa including the Araneoidea, Phyxelididae, and other members of the RTA clade. It is also

457 possible that they have evolved alternative uses for aciniform silk or do not need to produce this

458 silk until the adult instars when numbers of spigots on both spinnerets increase.

459   Sexual dimorphism was observed in PI gland and AC gland spigots in *D. tenebrosus* and

460   this has also been recorded in other lycosoids and araneid spiders (Wąsowska, 1977; Townley &

461   Tillinghast, 2009; Dolejš et al., 2014). We were not able to rear our *H. carolinensis* males to

462   adulthood in the lab colony, but given the trends in other Lycosidae, we would expect to observe

463   sexual dimorphism as well (Dolejš et al., 2014). In most spiders, regardless of lineage or

464   cribellate or ecribellate status, adult males lose (abort) spigots of all types in the final molt: this is

465   likely due to the shift in life history strategy of abandoning webs or territories to actively forage

466   and look for females (Wąsowska, 1977; Yu & Coddington, 1990; Hajer, 1991; Townley &

467   Tillinghast, 2009; Dolejš et al., 2014; R. Carlson & C.E. Griswold, unpublished data; R.E. Alfaro,

468   unpublished data).

469   Adult females of both species possessed a potential modified spigot (MS) on the PLS. A

470   modified spigot was not reported for other lycosoids in previous studies (Wąsowska, 1977;

471   Dolejš et al., 2014). Modified spigots have been reported as singular or, in some cases of

472   cribellate silk users among members of the RTA clade, which includes lycosoids, the MS may

473   occur with flanking spigots (Griswold et al., 2005; R.E. Alfaro, unpublished data). It is possible

474   that this modified spigot in lycosoids could be homologous to the pseudoflagelliform gland spigot

475   (PF) observed in cribellate lineages sister to the RTA clade, e.g., *Phyxelida tanganensis* (E.

476   Simon & L. Fage, 1922) and *Hyptiotes paradoxus* (C.L. Koch, 1834) (Table S1; Peters, 1984;

477   Peters, 1995; Griswold et al., 2005; Eberhard, 2010; Eberhard & Barrantes, 2015; R. Carlson &

478   C.E. Griswold, unpublished data). In contrast, it should be noted that in species with modified

479   spigots, adult males typically possess an MS nubbin, which we did not observe in male *D.*

480   *tenebrosus* (Ramírez, 2014, Table 5). To confirm these observations in *D. tenebrosus* and *H.*

481   *carolinensis*, replicate adult female and male specimens are necessary.

482   **Phylogenetic comparative analyses:**

483 Six analyses had significant correlation coefficients after correcting for the bias of shared

484 evolutionary history, suggesting that Instar, Strategy, and Type (spigot variety) are good

485 predictors of spigot number in spiders (Table 5). Although most of the PGLS analyses found no

486 significant correlation of the five independent variables with the 12 dependent variables of spigot

487 numbers, this is not altogether surprising considering the PGLS analyses remove the bias of

488 correlation due to shared evolutionary history. It is possible that with broader taxon sampling

489 deeper in the phylogeny, trends may emerge beyond those explained by phylogenetic signal. As

490 our analyses currently cannot include as a variable the full ontogeny dataset per species, it is also

491 more likely with future developments of more complex statistical analyses within a phylogenetic

492 context that will allow us to include the full ontogeny picture of each species, we will be able to

493 gain a better understanding of what is driving silk spigot evolution in spiders.

494   Maximum number of instars (Instar) served as a proxy for body size or body condition in

495 each spider species. Within a species with variation in maximum instar, we observed higher

496 numbers of spigots in the individuals of older instars (Table S1). Finding a significant correlation

497 between Instar and Average number of spigots in adult female aciniform and piriform gland

498 spigots is not unexpected, considering these spigots increase in number with each instar (Table

499 S1, Table 5). The more nutrition a juvenile spider consumes in one instar influences how much

500 growth occurs in the molt to the next instar. Spiders with a steady food supply may invest in an

501 increased number of instars to ensure better body condition at the adult stage, which could lead to

502 a trend of increase in spigot numbers (R.E. Alfaro, unpublished data). Piriform gland spigot

503 numbers were also positively correlated with Type (variety of spigots possessed) (Table 5).

504 Piriform gland silk is used as a cement for other silk fibers, particularly the major ampullate

505 gland fibers in dragline silk (Garb, 2013). In orb weavers, piriform gland silk is used to cement

506 the structural lines together and to the substrate whereas in wandering spiders it cements the

507   dragline to the substrate to prevent the spider from falling (Garb, 2013). Orb weavers and

508   lycosoids possess different types of silk spigots and have different uses for the shared spigots

509   they possess (Table S1, Table 2); for example, MAP gland silk may be used to construct aerial

510   frames (orb weavers) vs. surface dragline (lycosoids); aciniform gland silk may be used as web

511   material in wolf spiders who spin funnel webs (the genus *Hipassa*) rather than as a prey wrapping

512   material (orb weavers) (Mathew et al., 2011; Garb, 2013). It is possible that these differences in

513   use are due to the types of spigots they possess and their differing foraging strategies (web

514   building vs. predominately active hunting) and are what is causing this positive correlation we

515   observed (Table 5). Finally, in adult female PGLS analyses, Strategy (web vs. non-web) was a

516   significant predictor of the number of mAP gland spigots on the PMS. The coefficient of

517   correlation was small, but when we look at the full ontogeny data (Table S1), we see a clear

518   differentiation between araneoids and the others. In the adult female stage, araneoids lose one

519   mAP gland spigot and retain one functional spigot, whereas in the other groups, from the

520   lycosoids to the cribellate web builders, all female spiders retain the two mAP gland spigots that

521   they possessed throughout their ontogeny (Table S1). The clear correlation between strategy as a

522   predictor and mAP number as a response is expected since all araneoids possess one mAP spigot

523   and the remaining spider groups possess two (Correlation coefficient: 0.291, t = 2.448, p = 0.024:

524   Table 5). This may have withstood the phylogenetic correction of the PGLS, because several of

525   the non-araneoids within our study also spin webs.

526   The PGLS analyses for second instars were largely non-significant. The lack of

527   significant correlation between predictor and response variables may be due to second instars

528   being more similar. Many spiders start out with the same general number of spigots upon

529   emergence from the egg sac (second instar) and differentiation between foraging strategies may

530   not be apparent at this instar, e.g., some second instar web builders do not spin webs (Table S1,

531   Hajer et al., 1991; Barrantes & Madrigal-Brenes, 2008; R.E. Alfaro, unpublished data).

532   However, both MAP gland spigots on the ALS and mAP gland spigots on the PMS were

533   significantly correlated with Instar (maximum number of instars within each species) (Table 2,

534   Table 5). This correlation with Instar is likely a case where having the full ontogeny incorporated

535   into an analysis would provide clarity on this odd result. In general, web builders tended to have

536   less number of instars to adulthood than non-web builders or wandering spiders; exceptions in

537   this study are the ambush thomisid and sit & pursue (Miller et al., 2013) philodromid species:

538   *Xysticus cristatus* (C. Clerck, 1757) and *Tibellus oblongus* (C.A. Walckenaer, 1802), respectively

539   (Table S1), which had relatively few instars. Some second instars possess the two MAP gland

540   spigots observed in all species later in ontogeny. However, both *X. cristatus and T. oblongus* had

541   no MAP gland spigots in the second instar and *Metellina segmentata* (C. Clerck, 1757) possessed

542   only 1 MAP gland spigot (Table S1; Wąsowska, 1977; Yu & Coddington, 1990; Townley &

543   Tillinghast, 2009). These three species had some of the lower maximum numbers of instars per

544   species compared to the lycosoids, cribellate spiders and even viscous orb weavers (Table 2).

545   Second instars across species possessed either none, one or two mAP gland spigots and this

546   varied across foraging strategies and lineages. However, those species that possess both mAP

547   gland spigots at the second instar were consistently the species with a higher maximum number

548   of instars. This explains the significance of Instar as a predictor (Table S1).

549   **Ancestral character estimation**:

550   We also conducted an ancestral character estimation on specific spigots on the posterior lateral

551   spinneret whose potential homology have long been debated (Fig. 3; Peters, 1984; Peters, 1995;

552   Griswold et al., 2005; Eberhard, 2010; Eberhard & Barrantes, 2015; R. Carlson & C.E. Griswold,

553   unpublished data; R.E. Alfaro, unpublished data). We added an additional five species, including

554   two species sister to the Araneoids, and three cribellate species ancestral to both the Araneoidea

555   and RTA clades (Fig. 3). As we previously described we constrained our rate matrix (Table 6)

556   that we used to model substitution rates across branch lengths based on prior knowledge about

557   historical possession of the flagelliform, pseudoflagelliform and modified spigots in each taxon

558   and considering Dollo's Law (Table 6) (Dollo, 1893; Platnick et al., 1991; Griswold et al., 2005;

559   Dimitrov et al., 2016; Wheeler et al., 2016).

560   The ancestral root of our phylogram of 27 species was more likely to have borne a

561   modified spigot or none at all (likelihood: 0.365, 0.635, respectively) (Fig. 3, Results S5).

562   Because this is not a full determination of the likelihood of a modified spigot or no spigot we

563   cannot definitively determine the character state of the orb weaving ancestor at the node where

564   Nicodamoidea + Araneoidea and the RTA clade split off (Fig. 3, Modified spigot likelihood:

565   0.398, None likelihood 0.602). It was unlikely to have possessed a pseudoflagelliform gland

566   spigot (Fig. 3, Pseudoflagelliform likelihood: 0.000). We can, therefore, hypothesize that

567   pseudoflagelliform and modified spigots are homologous structures and that modified spigots in

568   the RTA clade likely are retained structures like those found in the sister and ancestral cribellate

569   clades (Fig. 3). We cannot rule out that flagelliform gland spigots arose independently from

570   modified spigots and thus cannot infer homology between this spigot and the others. We do not

571   know the functionality of the modified spigots observed in *D. tenebrosus* and *H. carolinensis*.

572   This would be useful to explore in the future, as the functionality of modified spigots in cribellate

573   members of the RTA clade is the same as the pseudoflagelliform gland spigot (R.E. Alfaro et al.,

574   unpublished data). It is possible we are observing an intermediate stage of the loss of the

575   modified spigot in *D. tenebrosus* and *H. carolinensis*.

576   Our results may change if we can incorporate ontogeny into the ACE analysis. For

577   example, mimetids, which were ranked as having no spigots, do possess vestigial PLS spigots in

578   the early instars (Table S1, Townley & Tillinghast, 2009). It is also possible that we are

579   observing in real time the loss of the flagelliform gland spigot in this araneoid lineage. *T.*

580   *perfuga* possess primordial modified spigot and flankers in second instars prior to them

581   constructing webs in the third instar where functional spigots exist where the pre-spigots had

582   been (Table S1; R.E. Alfaro, unpublished data). In most species, the final molt of the adult male

583   leads to loss or nubbins (non-functional spigots) in all three: Modified, Pseudoflagelliform,

584   Flagelliform (Table S1; Wąsowska, 1977; Yu & Coddington, 1990; Hajer, 1991; Townley &

585   Tillinghast, 2009; Dolejš et al., 2014; R. Carlson & C.E. Griswold, unpublished data). This

586   coincides with the male abandonment of the web for an alternative lifestyle of wandering in order

587   to find females.

588   Future work, as the methods of phylogenetic inference grow and progress, may yield

589   different results if we incorporate this analysis with the triad of spigots, i.e., the triplet of MS plus

590   flankers, PF and flankers and/or FL (flagelliform) plus AG (aggregate), or spigot associations, i.e.

591   PC with the PF in cribellate orb weavers. In araneoids, the flagelliform gland spigot is flanked by

592   two aggregate gland spigots and in *T. perfuga* the modified spigot is flanked by two spigots of

593   unknown gland association. In *Tengella,* these flankers resemble AC gland spigots but in some

594   other cribellate spiders, e.g., *Matachia* or *Badumna* (Griswold et al., 2005; figs 87 A, D) the

595   flankers resemble paracribellar (PC) gland spigots. The color-coded ACE phylogram shows a

596   trend of loss (black color) for all spigot types in approximately half of the tip species (Fig. 3).

597   This coincides with a shift in foraging strategies, i.e., from webs to running, observed in these

598   lineages (Table 2) and is consistent with the current hypothesis that an adaptive tradeoff between

599   silk production and fecundity is driving spider evolution to foraging strategies that do not involve

600   silk or web building (Blackledge et al., 2009). Given the recent conclusions of phylogenomics

601   studies indicating a much more ancient orb weaving ancestor and the new sister relationship of

602   cribellate orb weavers to the RTA clade, our ACE results do indicate that deeper and broader

603   sampling across the spider tree of life is necessary (Bond et al., 2014; Fernández et al., 2014;

604   Garrison et al., 2014).

605   Increased taxon sampling would also improve the ancestral character estimation analyses

606   performed for the independent variables of the PGLS analyses. Not surprisingly, given the

607   results and discussion of the above analysis, the ancestral state at the root node of the phylogram

608   of 27 species was Web builder for Strategy [foraging strategy; likelihood = 1.0], and specifically

609   either funnel (likelihood = 0.812), sheet (likelihood = 0.084) or orb webs (likelihood = 0.105)

610   (Figs 9, 10). Also, given the ancestral state for the singular spigot on the PLS as Modified spigot

611   (MS), one may reason that the ancestor was also a cribellate silk user. We found this to be the

612   case after performing ACE on the independent variable Silk (main type of silk expressed), and

613   found the ancestral state at the root of the phylogram was 100% likely to be cribellate (Fig 11).

614   Finally, Type or variety of spigot types, was less conclusive and we found likelihood values for

615   all states at the root node (Table S2). However, the most likely ancestral states were those

616   possessing a cribellum, paracribellum (likelihood = 0.398) and possibly a pseudoflagelliform

617   gland spigot (likelihood = 0.433) (Fig 12). This may not actually conflict with our results for the

618   ACE of the singular PLS spigot, i.e., that MS is ancestral, in that we determined that the MS and

619   PF spigots are homologous structures. But we also found that the most likely state was no

620   modified spigot (likelihood = 0.635), which would suggest the plesiomorphic state of cribellum +

621   paracribellum, without any modifications of spigots on the PLS is the ancestral state at the root

622   node of the ultrametric phylogram.

623   As the techniques for more complex phylogenetic comparative analyses improve, such as

624   to allow for a time-series dataset with multiple values per species, we suspect that incorporating

625   the entire picture of spigot ontogeny will lead to some interesting and novel inferences about silk

626   evolution. By not incorporating the entire ontogeny, but "snapshots" of the adult female and

627   second instars, important observations are missed by the analyses, such as loss and regain of AC

628   gland spigots on the PLS in *H. carolinensis,* or presence of FL-AG triad spigots in early

629   Mimetidae instars. This approach to understanding spigot ontogeny from a phylogenetic

630   comparative perspective is novel and we can only build on our efforts from this study by growing

631   the dataset to include deeper taxon sampling and working towards the capability of phylogenetic

632   statistical analyses that can function to accommodate ontogeny datasets as whole units for each

633   species.

634   **Conclusions:**

635   In this study we explored spigot ontogeny in 22 species, including novel observations of

636   *Dolomedes tenebrosus* and *Hogna carolinensis*. This is the first effort to create a phylogenetic

637   comparative approach utilizing the recent Araneae Tree of Life. We performed 60 PGLS

638   analyses of five independent variables: Strategy, Specific, Silk Type, Instar and twelve dependent

639   variables (spigot numbers in adult females and second instars juveniles). Six had significant

640   correlation coefficients indicating Instar, Strategy and Type (spigot variety) as good predictors of

641   spigot numbers in spiders. Next, after adding five additional spider taxa to allow for deeper and

642   broader taxon sampling within the Araneomorphae, we reconstructed ancestral character

643   estimations of the unique, singular silk spigots on the PLS whose potential homology has been

644   debated. The analysis predicted the ancestral root to either have no singular spigot or to possess a

645   modified spigot (MS). Finally, additional ACE analyses on four of the five independent variables,

646   suggest that the ancestral root of the RTA clade and Araneoidea was likely a spider that was

647   cribellate, spun a web, and possessed a more diverse array of spigots. We also suggest that the

648   modified spigot (MS) and pseudoflagelliform gland spigots (PF) are homologous structures.

649   Current statistical methods do not allow for multiple values for species within PGLS, which

650 limited our ontogenetic scope. However, as methods allow for more complex datasets, by

651 looking at the full picture of spigot morphology during a spider life cycle across multiple clades

652 of the AToL, we should be able to gain more depth of our understanding of silk evolution in

653 spiders.

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Full spigot ontogeny of *Dolomedes tenebrosus* and *Hogna carolinensis*.

Numbers of functional spigots by spinneret for each instar. Pre-spigots are noted for those whose functionality is determined. * Indicates a tentative identification which requires more replicates to confirm.

| Species | Instar | Spinneret | | | | | | | |
| | | ALS | | PMS | | | PLS | | |
		MAP	PI	mAP	AC	CY	AC	CY	Modified
Dolomedes tenebrosus	*2*	2	6	2	4	0	4	0	0
Hogna carolinensis	*2*	2	4	2	4	0	7	0	0
Dolomedes tenebrosus	*3*	2	9	2	4	0	6	0	0
Hogna carolinensis	*3*	2	7	2	6	0	9	0	0
Dolomedes tenebrosus	*4*	2	9	2	5	0	6	0	0
Hogna carolinensis	*4*	2	11	2	3	0	3	0	0
Dolomedes tenebrosus	*5*	2	14	2	5	0	6	0	0
Hogna carolinensis	*5*	2	13	2	3	0	3	0	0
Dolomedes tenebrosus	*6*	2	16	2	5	0	9	0	0
Hogna carolinensis	*6*	2	17	2	6	0	7	0	0
Dolomedes tenebrosus	*7*	2	18	2	6	0	8	0	0
Hogna carolinensis	*7*	2	27	2	4	0	7	0	0
Dolomedes tenebrosus	*8*	2	27	2	8	0	9	0	0
Dolomedes tenebrosus	*9*	2	57	2	8	0	15	0	0
Dolomedes tenebrosus	*10 antepen-♀*	2	52	2	8	0	10	0	0
Dolomedes tenebrosus	*11 pen-♂*	2	75	2	9	0	14	0	0
Dolomedes tenebrosus	*12 pen-♀*	2	133	2	30	Pre1	42	Pre3	Pre1
Dolomedes tenebrosus	*12 ♂*	1	54	1	7	0	13	0	0
Hogna carolinensis	*12 ♀*	2	122	2	82	10	43	1	1*
Dolomedes tenebrosus	*13 ♀*	2	107	2	15	32	24	28	1

Species included in PGLS analyses by spider family.

Twenty-two species across thirteen spider families were used in the PGLS analyses. The scoring for the five independent variables, from Foraging Strategy to Maximum Number of Instars is reported here. 'Strategy' delineates web (0) or no web usage (1). 'Specific' refers to the type of prey pursuit (1-3) or web (4-5) used by each species. 'Silk' quantifies the main type of silk used by each species from aciniform (1) to viscous (4). 'Type' is a numerical designation for the variety of silk spigot types each species possesses from the 'standard 7' (1) to standard 7 + paracribellar + cribellum + pseudoflagelliform (4). Finally, 'Instar' refers to the maximum number of instars reported for adult females of each species.

Family	Species	Strategy	Specific	Silk	Type	Instar
Philodromidae	*Tibellus oblongus*	1	2	1	1	6
Thomisidae	*Xysticus cristatus*	1	1.5	1	1	6
Lycosidae	*Xerolycosa nemoralis*	1	3	1	1	10
	Pardosa lugubris	1	3	1	1	7
	Pardosa amentata	1	3	1	1	9
	Hogna carolinensis	1	2	1	2	12
	Arctosa lutetiana	1	1.5	1.5	1	9
	Arctosa alpigena lamperti	1	3	1	1	10
Pisauridae	*Dolomedes tenebrosus*	1	1	1	2	13
Zoropsidae	*Tengella perfuga*	0	4.5	3	2.5	12
Dictynidae	*Argyroneta aquatica*	0	4	2	1	6
Agelenidae	*Eratigena atrica*	0	4.5	2	1	9
Phyxelididae	*Phyxelida tanganensis*	0	4	3	4	8
Uloboridae	*Hyptiotes paradoxus*	0	5	3	4	6
Tetragnathidae	*Metellina segmentata*	0	5	4	3	5
Mimetidae	*Mimetus puritanus*	1	2.5	1	1.5	7
	Mimetus notius	1	2.5	1	1.5	7
Araneidae	*Neoscona theisi*	0	5	4	3	7
	Araneus cavaticus	0	5	4	3	12
	Araneus diadematus	0	5	4	3	10
	Larinioides cornutus	0	5	4	3	7
Theridiidae	*Enoplognatha ovata*	0	5.5	4	3	4

Results of tests for phylogenetic signal.

Results of the tests for phylogenetic signal using Pagel's λ and Blomberg's K for all independent and dependent variables. Those with significant phylogenetic signal are bolded and include all independent variables and three of the response variables.

	Variable	Pagel's λ	P-value	Blomberg's K	P-value
(I)	**Strategy**	**1.000**	**0.0006**	**0.955**	**0.001**
(I)	**Specific**	**1.000**	**0.002**	**0.904**	**0.002**
(I)	**Silk**	**1.000**	**0.0003**	**0.915**	**0.001**
(I)	**Type**	**1.000**	**0.00008**	**1.243**	**0.001**
(I)	Instar	0.565	0.139	**0.512**	**0.012**
(D)	**Fem ALS MAP**	**1.000**	**0.00004**	**0.634**	**0.009**
(D)	Fem ALS PI	0.110	0.698	0.158	0.795
(D)	**Fem PMS mAP**	**1.000**	$5.36\ e^{-10}$	**3.834**	**0.002**
(D)	Fem PMS AC	0.532	0.210	0.176	0.769
(D)	Fem PMS CY	0.229	0.141	0.452	0.076
(D)	Fem PLS AC	0.572	0.270	0.184	0.731
(D)	Fem PLS CY	0.057	0.698	0.315	0.243
(D)	2nd ALS MAP	6.61E-05	1.000	0.313	0.287
(D)	2nd ALS PI	0.076	0.603	0.093	0.877
(D)	**2nd PMS mAP**	**1.000**	**0.002**	**0.794**	**0.008**
(D)	2nd PMS AC	6.61E-05	1.000	0.084	0.871
(D)	2nd PLS AC	6.61E-05	1.000	0.081	0.895

Significant results of Model Selection for PGLS analyses.

Model selection results for selected single-term PGLS models, showing Instar as most important and the model selection as significant. AIC_c and ΔAIC_c values for model selection for all independent variables, by response variable. Term codes are: Instar = 1, Type = 2, Silk = 3, Specific = 4, Strategy = 5. Significant results are bolded, if not significant, but most important term, they are bold italicized.

2 **2nd Instar ALS MAP Model Selection:**

Term	AIC$_C$	Δ	Weight
Instar	**43.35**	**0.00**	**0.84**
Strategy	48.75	5.40	0.06
Type	49.32	5.97	0.04
Specific	49.35	5.99	0.04
Silk	50.57	7.21	0.02

4 **2nd Instar ALS Piriform Model Selection:**

Term	AIC$_C$	Δ	Weight
1	*205.31*	*0.00*	*0.37*
3	206.80	1.49	0.18
5	207.03	1.72	0.16
4	207.13	1.82	0.15
2	207.15	1.83	0.15

6 **Female PMS Aciniform Model Selection:**

Term	AIC$_C$	Δ	Weight
1	**258.04**	**0.00**	**0.98**
2	268.36	10.32	0.01
5	268.51	10.47	0.01
3	268.70	10.66	0.00
4	268.74	10.70	0.00

Female ALS Piriform Model Selection:

Term	AIC$_C$	Δ	Weight
1	**238.30**	**0.00**	**1.00**
2	255.39	17.09	0.00
5	258.27	19.98	0.00
3	259.17	20.88	0.00
4	260.27	21.97	0.00

Female PLS Aciniform Selection:

Term	AIC$_C$	Δ	Weight
1	**251.95**	**0.00**	**0.91**
4	259.03	7.08	0.03
5	259.18	7.23	0.02
3	259.58	7.62	0.02
2	259.83	7.88	0.02

Significant results of PGLS analyses.

Significant results of the phylogenetic generalized least squares analyses testing for correlation of independent variables with the twelve dependent variables (average number of each standard spigot) for both adult female datasets and second instar datasets. The coefficient values provide the correlation coefficient of the means of the independent variable with the dependent variable.

Second Instar ALS MAP

Model: Average ~ Instar

PGLS Coefficient:		t	P
Instar	0.095	2.283	0.034

Second Instar PMS mAP

Model: Average ~ Instar

PGLS Coefficient:		t	P
Instar	0.104	2.641	0.016

Female PMS mAP

Model: Average ~ Strategy

PGLS Coefficient:		t	P
Strategy	0.291	2.448	0.024

Female ALS Piriform

Model: Average ~ Instar

PGLS Coefficient:		t	P
Instar	17.204	5.355	<0.000

Model: Average ~ Type

PGLS Coefficient:		t	P
Type	61.692	4.413	0.0003

Female PMS Aciniform

Model: Average ~ Instar

PGLS Coefficient:		t	P
Instar	11.725	2.857	0.010

Evolutionary rate matrix for ACE of unique PLS spigots.

The substitution rate matrix for the spigots of the PLS used as the model for the ancestral character estimation analysis. The rows are the from direction, while the columns are the to direction for state changes.

SPIGOT	Flagelliform	Loss	Modified	None	Pseudoflagelliform
Flagelliform	--	1	0	0	0
Loss	0	--	0	0	0
Modified	1	1	--	1	1
None	1	0	1	--	1
Pseudoflagelliform	1	1	0	0	--

Figure 1

Scanning electron micrographs of a spider spinning apparatus morphology.

Spinning fields of *Dolomedes tenebrosu*s (A, whole field, 7[th]instar) and *Hogna carolinensis* (B, right field, 4[th]instar). ALS = Anterior lateral spinnerets, PMS = Posterior median spinnerets, PLS = Poster lateral spinnerets.
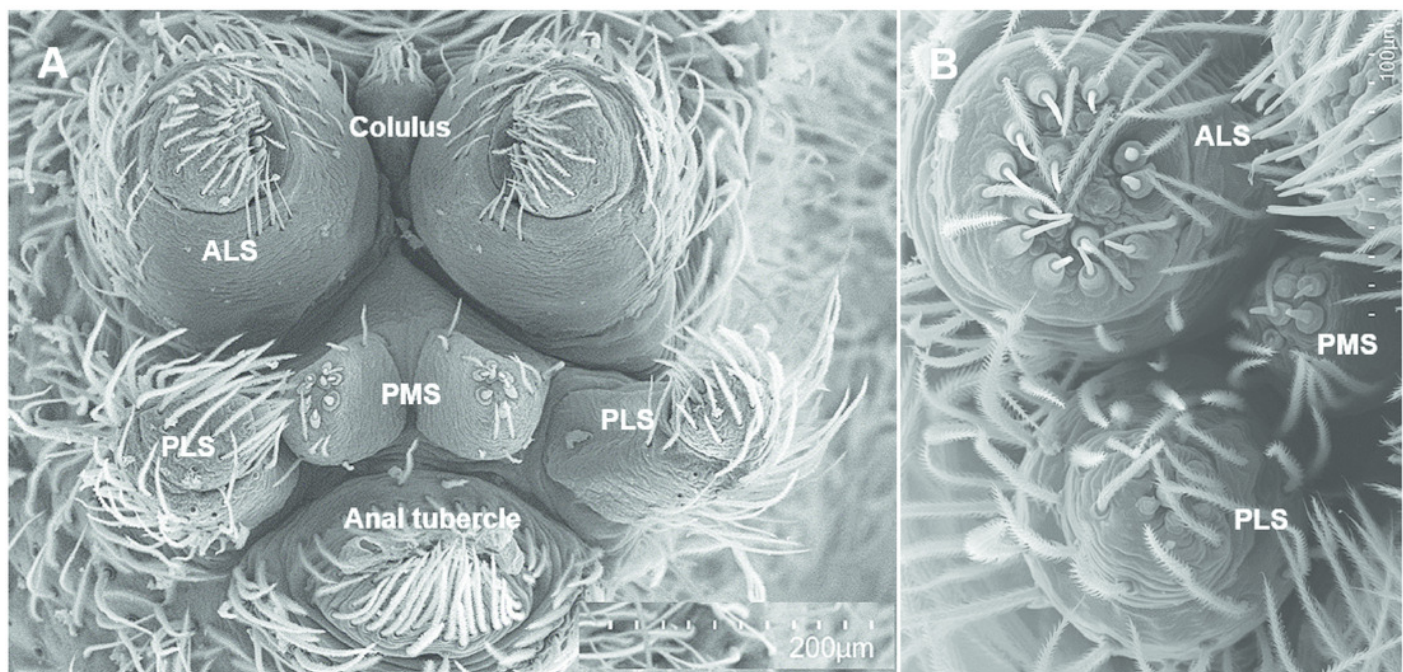
Figure 2

Phylogenetic tree used in PGLS analyses.

Ultrametric tree with 22 species, topology follows the AToL (Wheeler et al. 2016).
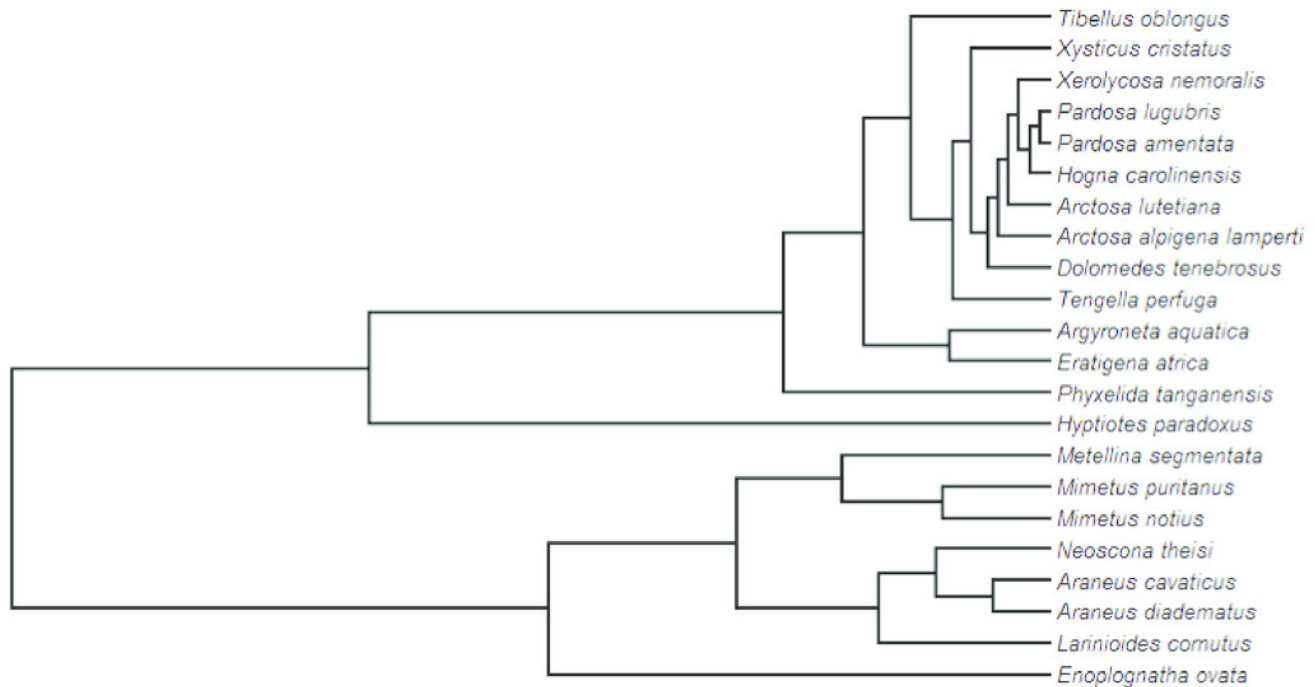
Figure 3

Ancestral character estimation results for the unique PLS spigots.

Phylogram with ancestral character estimation of singular PLS spigots on the ultrametric tree with five additional taxa. The root node is estimated to either possess a modified spigot (green) or no specialized spigots (blue).
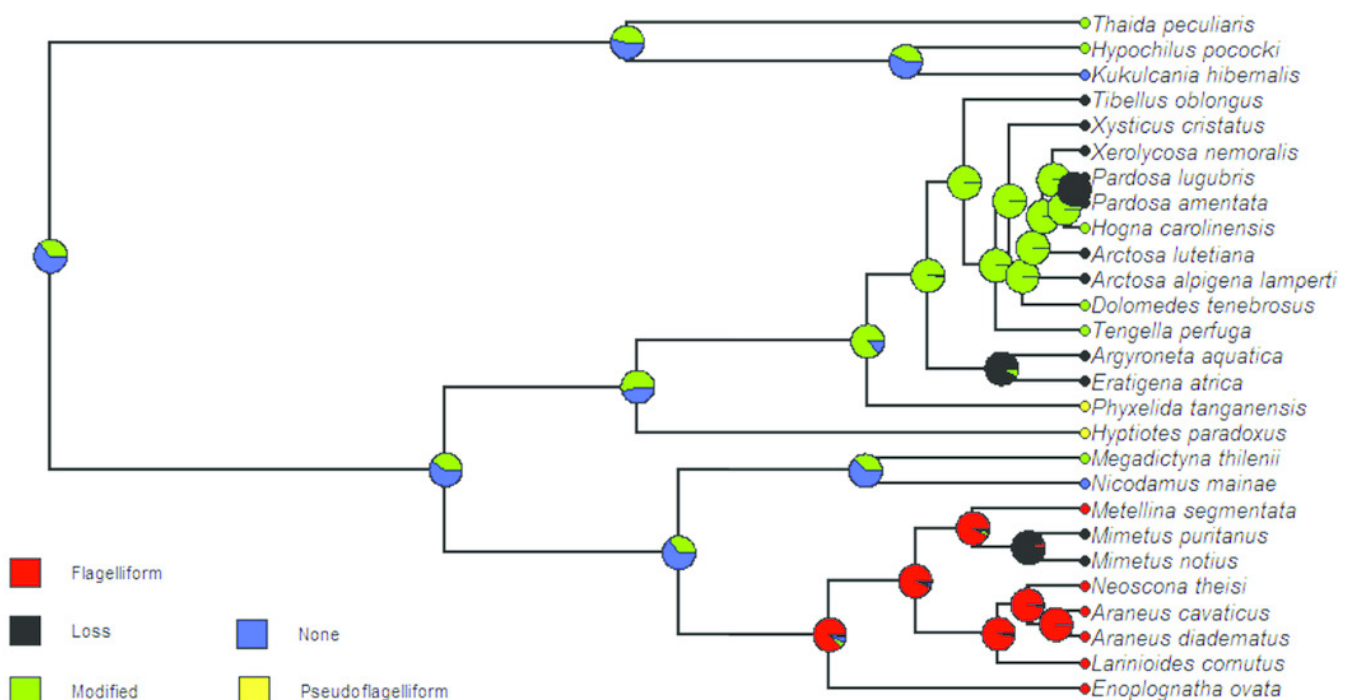
Figure 4

SEMs of the anterior lateral spinnerets.

ALS spinning field of selected instars of *Dolomedes tenebrosus* and Hogna *carolinensis*: A: *D. tenebrosus* 3rd instar left ALS; B: *H. carolinensis* 4th instar (left ALS); C. *H. carolinensis* 5th instar (left ALS); D. *D. tenebrosus* 8th instar (left ALS); E. *D. tenebrosus* 12th instar (right ALS, penultimate female). MAP = Major ampullate gland spigot.
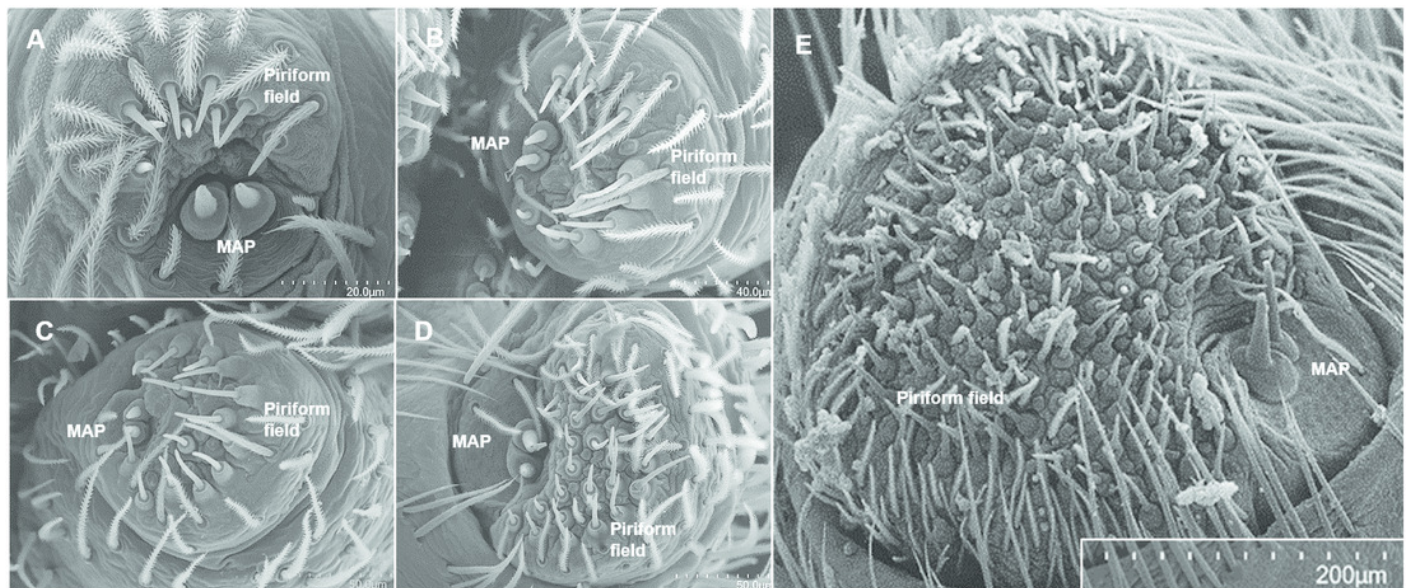
Figure 5

SEMs of the sensilla of the anterior lateral spinnerets.

Pore field of the MAP on the ALS of selected instars of *Dolomedes tenebrosus* and *Hogna carolinensis*: A. *H. carolinensis* 3rd instar (left ALS); B. *H. carolinensis* 5th instar (left ALS); C. *D. tenebrosus* 11th instar (penultimate male, left ALS); D. *D. tenebrosus* 12th instar (penultimate female, right ALS). MAP = Major ampullate gland spigot, Sensilla = sensory pores in MAP field.
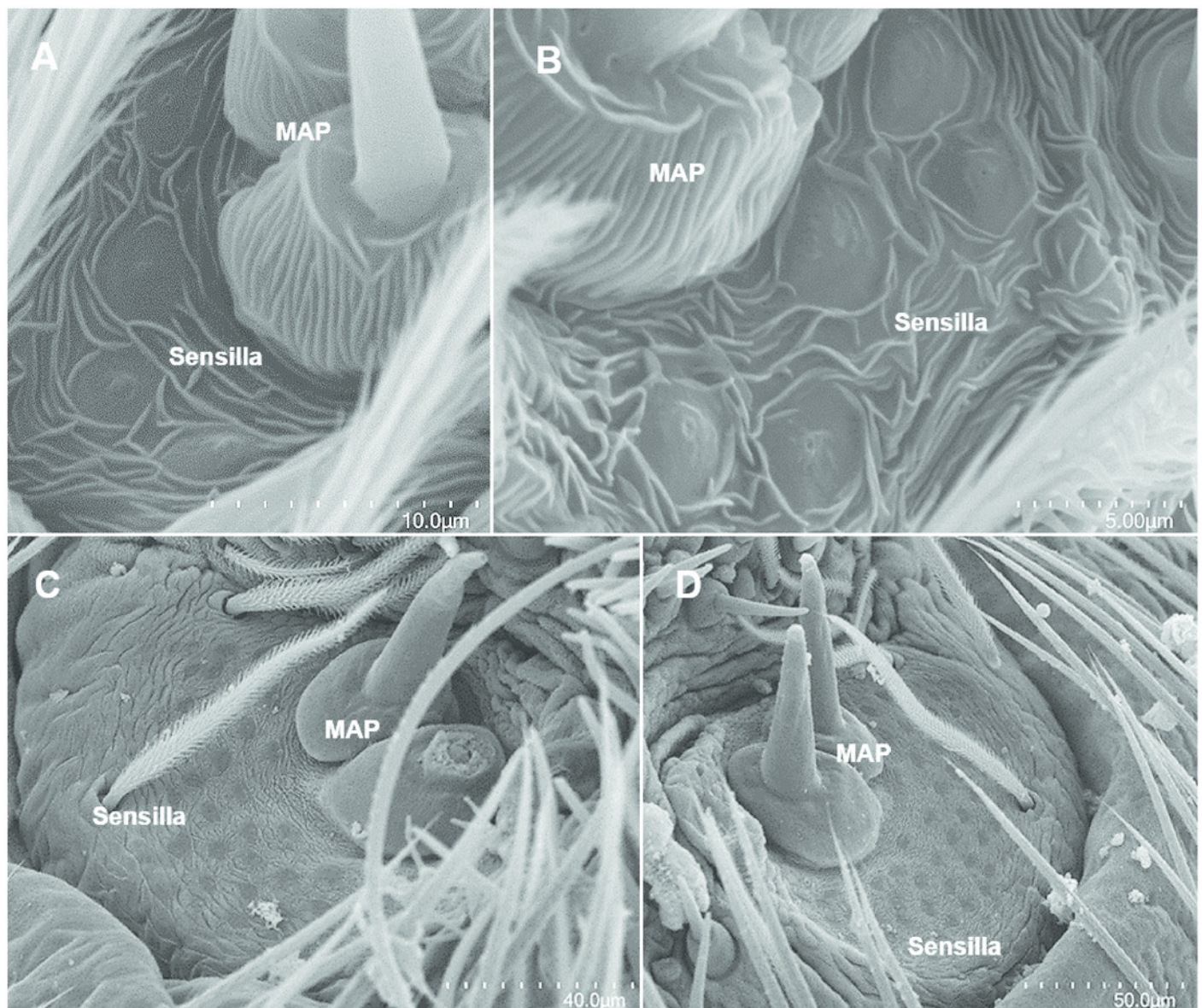
Figure 6

SEMs of the posterior median spinnerets.

PMS spinning field of selected instars of *Dolomedes tenebrosus* and *Hogna carolinensis*: A. *H. carolinensis* 2nd instar (left PMS); B. *H. carolinensis* 6th instar (right PMS); C. *H. carolinensis* 12th instar, female (left PMS); D. *D. tenebrosus* 3rd instar (left PMS); E. *D. tenebrosus* 5th instar (right PMS); F. *D. tenebrosus* 12th instar, penultimate female (right PMS). mAP = Minor ampullate gland spigot, Pre-CY = pre-cylindrical gland spigot.
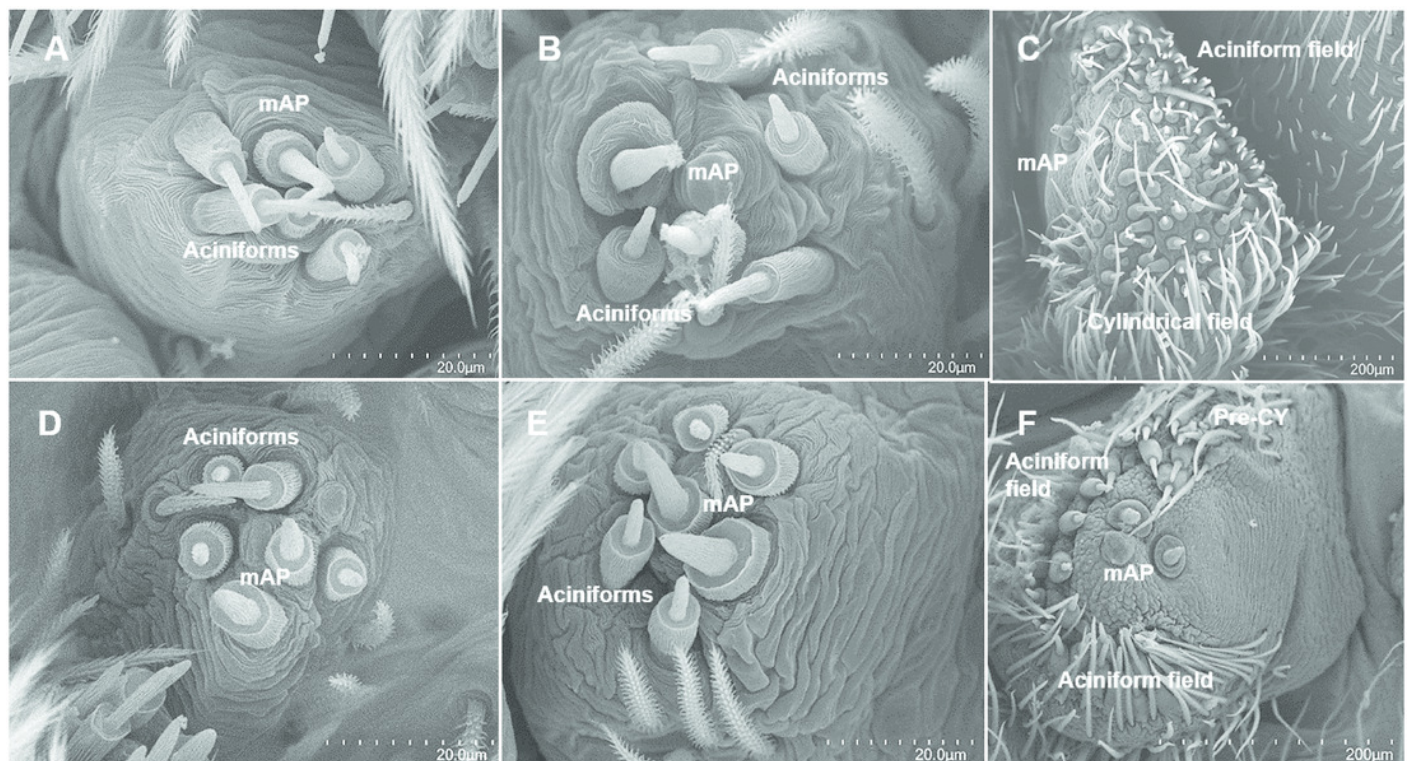
Figure 7

SEMs of the posterior lateral spinneret spigots of *Dolomedes tenebrosus*.

PLS spinning field of selected instars of *Dolomedes tenebrosus*, early instars have low conserved numbers of aciniform gland spigots, which suddenly increase at the penultimate instar: A. 3rd instar (left PLS); B. 4th instar (right PLS); C. 6th instar (left PLS); D. 8th instar (left PLS); E. 10th instar, antepenultimate female (left PLS); F. 12th instar, penultimate female (left PLS). Pre-CY = Pre-cylindrical gland spigots, Pre-Mod = Pre-Modified spigot.
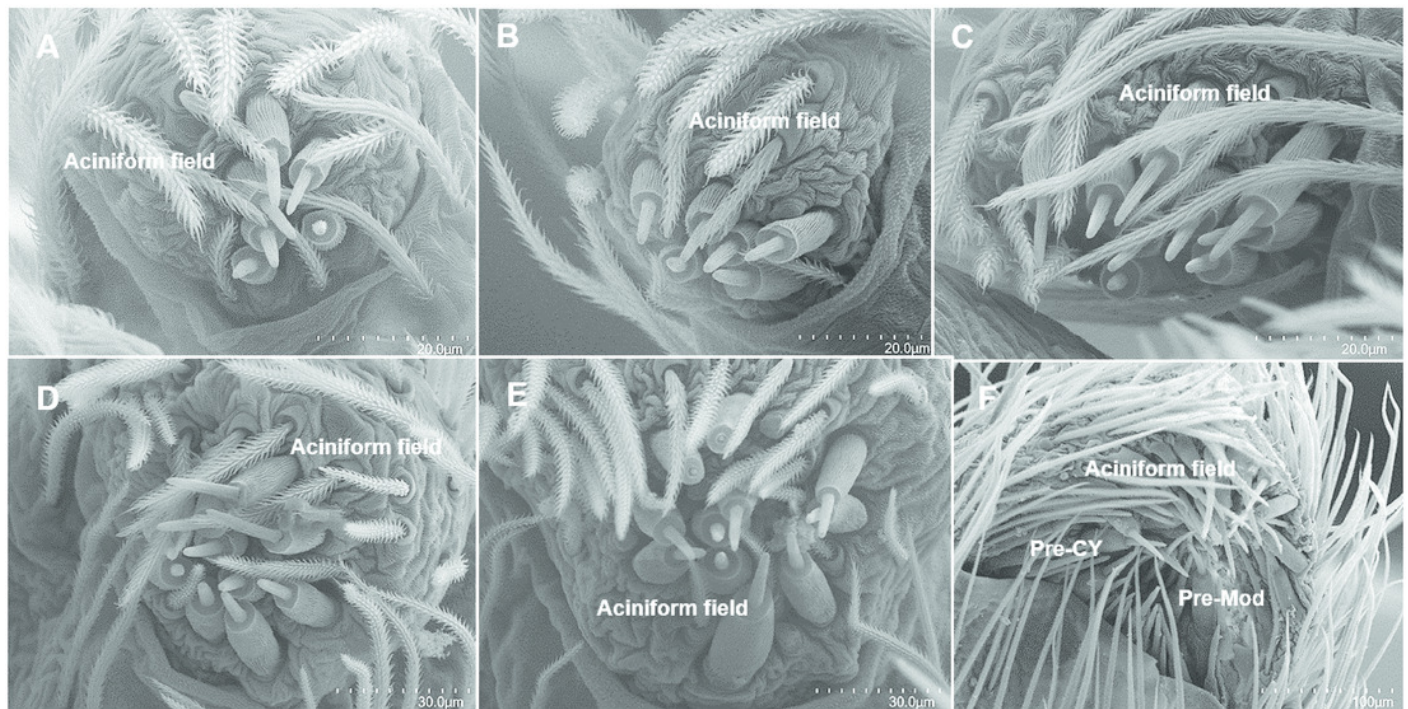
Figure 8

SEMs of the posterior lateral spinneret spigots of *Hogna carolinensis*.

Right PLS spinning field of selected instars of *Hogna carolinensis*, early instars have higher numbers of aciniform gland spigots, lose them over two molts, then begin to gain them back again: A. 2nd instar; B. 3rd instar; C. 4th instar; D. 5th instar; E. 6th instar. Aciniform tartipores = cuticular scars from aciniform gland spigots present in the previous instar.
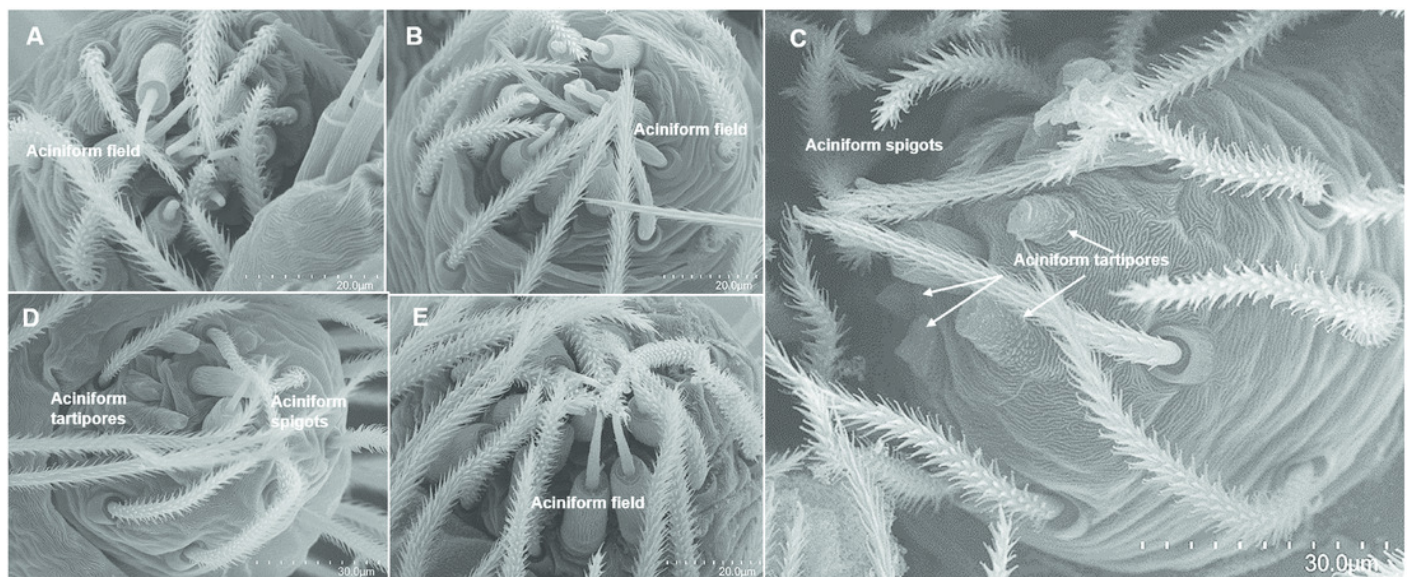
Figure 9

ACE results for foraging strategy.

Phylogram with ancestral character estimation of foraging strategy (Strategy), web or no web (loss) on the ultrametric tree with five additional taxa. Clearly, the ancestral state was web spinning (red).
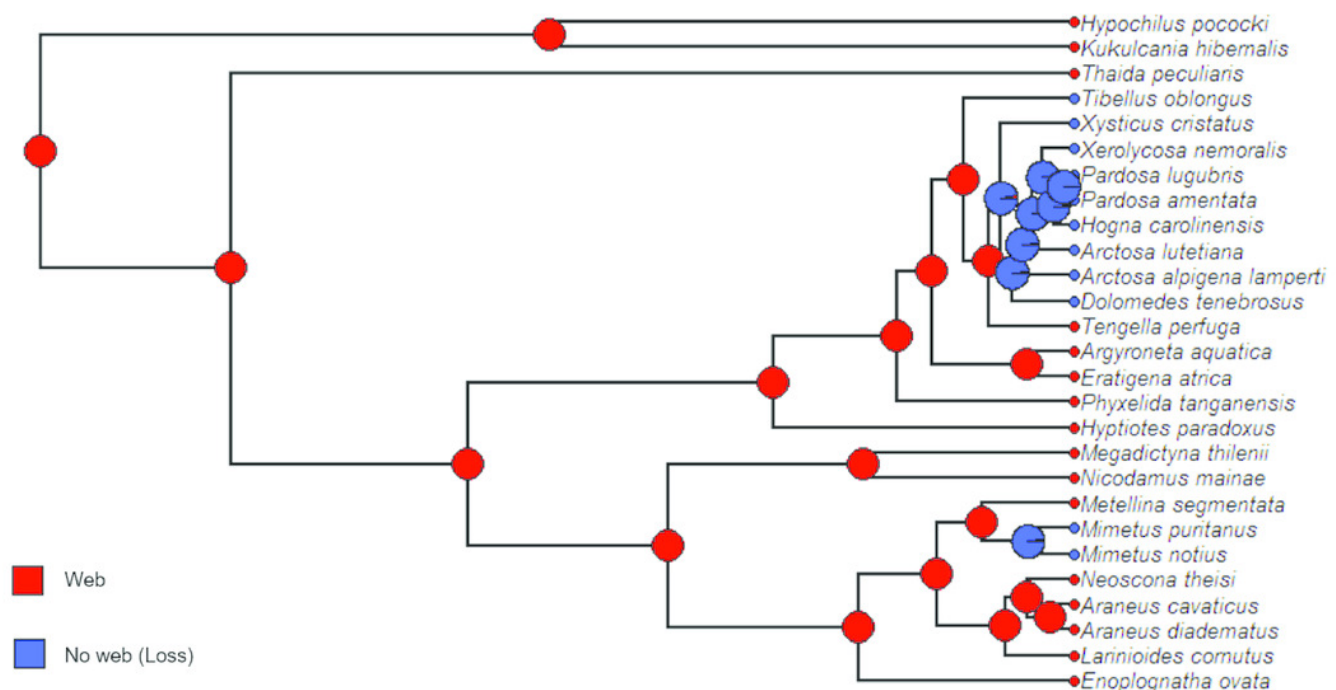
Figure 10

ACE results for specific foraging strategies.

Phylogram with ancestral character estimation of specific foraging strategies on the ultrametric tree with five additional taxa. Specific strategies include: sit & wait, ambush, sit & pursue, stalking, active, sheet web, funnel web, orb web and tangle web.
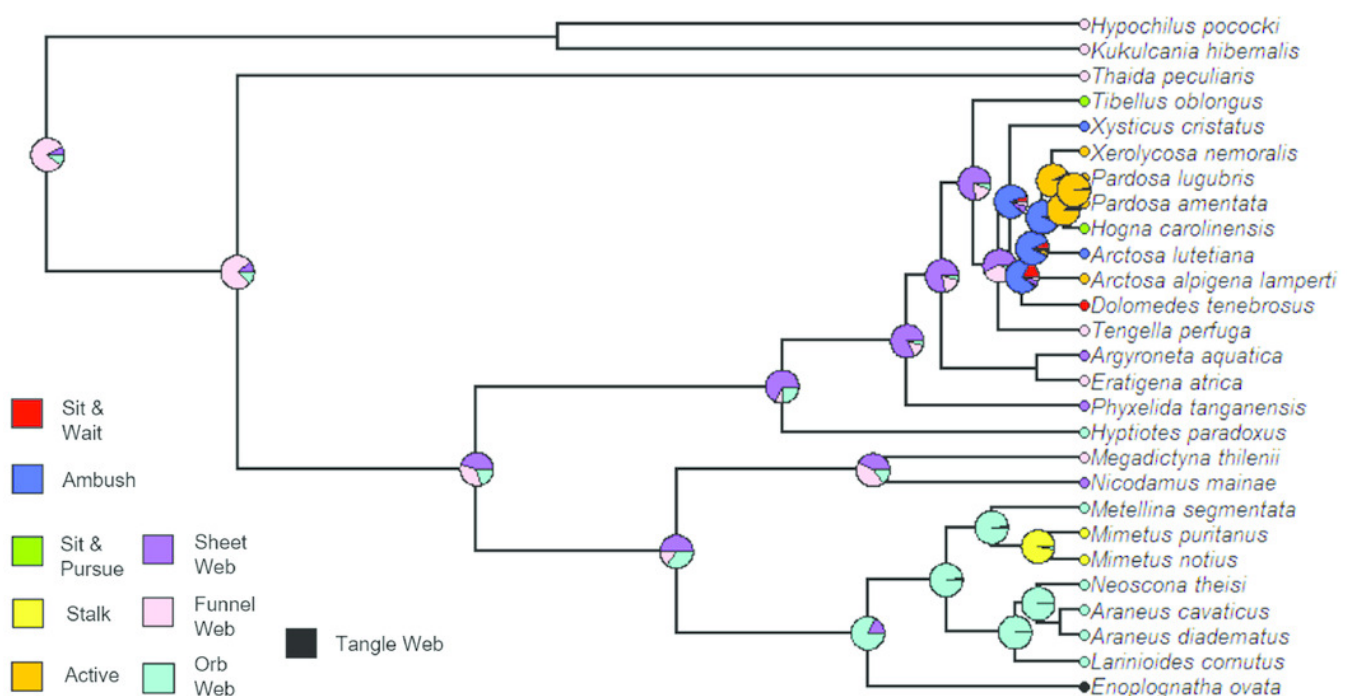
Figure 11

ACE results for main type of silk utilized by a species.

Phylogram with ancestral character estimation of Silk (main type of silk expressed) on the ultrametric tree with five additional taxa. Silk types include: none, burrow, aciniform, cribellate and viscous. This analysis suggests that the ancestral state for these clades is cribellate (yellow).
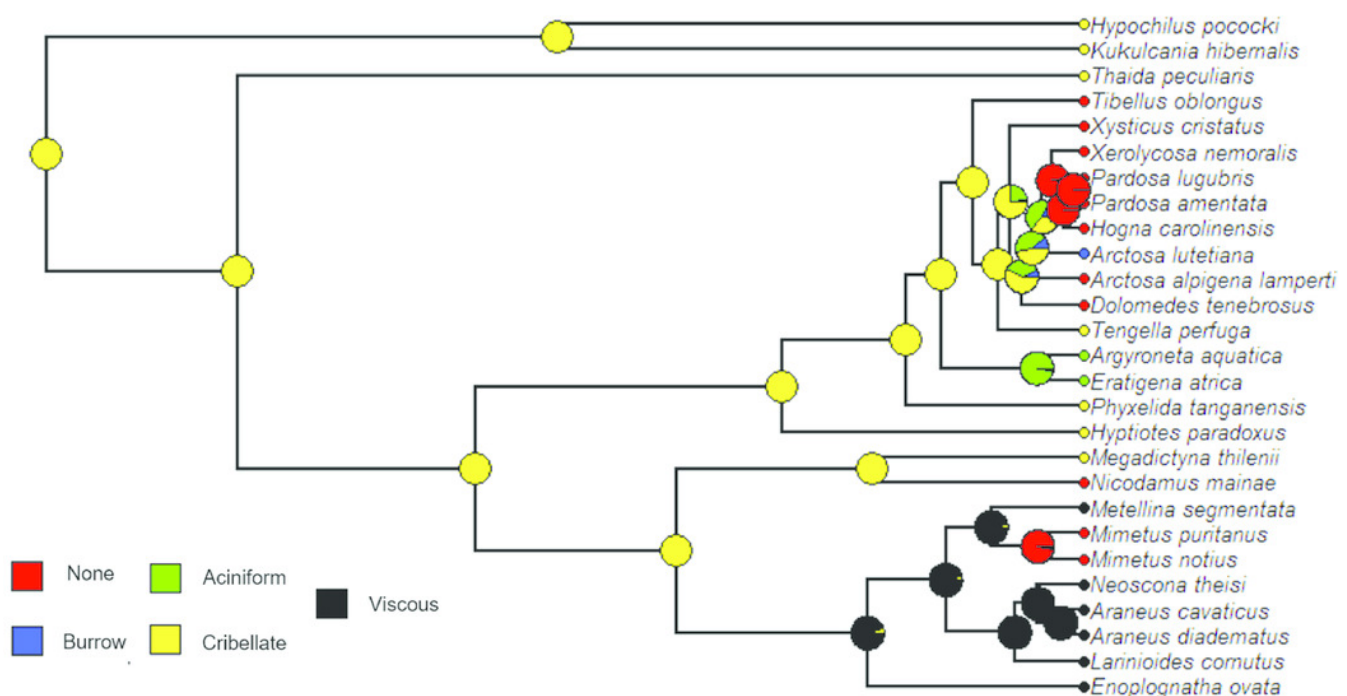
Figure 12

ACE results for variety of spigots possessed by a species.

Phylogram with ancestral character estimation of Type of spigots (variety of spigot types possessed) on the ultrametric tree with five additional taxa. These included several states, including the standard 7 spigots, along with additional spigots increasing in diversity. Std 7 = standard 7; MsPi = modified piriform gland spigots; MS = modified spigot; Crib = cribellum; Para = paracribellar spigots; Ag = aggregate gland spigots; Fl = flagelliform gland spigot; Pseudo = pseudoflagelliform gland spigot.