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Embryogenesis in a Colorado population of *Aphonopelma hentzi* (Girard, 1852) (Araneae: Mygalomorphae: Theraphosidae): establishing a promising system for the study of mygalomorph development

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Abstract. The mygalomorph spider *Aphonopelma hentzi* (Girard, 1852) (Texas brown tarantula) is a geographically widespread and accessible system for the study of comparative development in spiders. However, remarkably little information is available regarding the timing of egg sac deposition and duration of embryogenesis in this species, a gap that can directly affect the success of field collecting efforts. Here, we describe the habitat, egg sacs, and embryos of *A. hentzi* in the Comanche National Grasslands of Colorado. We compare the effectiveness of strategies for obtaining adult specimens vs. egg sacs of *A. hentzi* from burrows. Protocols for rearing, fixation, fluorescent immunohistochemistry, colorimetric *in situ* hybridization, and imaging of embryos are detailed. Together with forthcoming genomic resources, these data are anticipated to improve understanding of embryogenesis in Mygalomorphae and comparative development of chelicerates more generally.

Keywords: Ontogeny, opisthosoma, *Parasteatoda tepidariorum*, spider, spinnerets

The mygalomorph spider genus *Aphonopelma* Pocock, 1901 has featured prominently in recent systematic endeavors pertaining to phylogenetic relationships and species delimitation (e.g., Hamilton et al. 2011, 2016; Hendrixson et al. 2013, 2015; Graham et al. 2015). In such works, adult specimens are typically collected in the field, as species-specific characteristics are often most apparent in adulthood. Comparatively few published data are available on the reproductive cycle of *Aphonopelma*, outside of captive breeding efforts of hobbyists. Such data are essential to revitalizing the study of comparative development in spiders, as much of our knowledge of spider embryogenesis is restricted to entelegyne araneomorphs (e.g., Herold 1824; Wittich 1845; Strand 1906; Kautzsch 1909; Montgomery 1909; Holm 1941; Rempel 1957; Anderson 1973; Akiyama-Oda & Oda 2003, 2006; Wolff & Hilbrant 2011; Mittmann & Wolff 2012). Comparatively fewer embryological data are available for "haplogynes" (Holm 1941; Turetzek & Prpic 2016; Turetzek et al. 2017) and mygalomorphs (Schimkewitsch & Schimkewitsch 1911; Holm 1954; Yoshikura 1958; Pechmann & Prpic 2009), and almost none for mesotheles (Yoshikura 1955). The development of non-araneomorph spiders thus is largely unknown outside of a few descriptive works (Anderson 1973; Pechmann et al. 2010; Turetzek & Prpic 2016). This is partly attributable to the typically long generation times of groups like Mygalomorphae (measured in years; by comparison, the theridiid *Parasteatoda tepidariorum* (C.L. Koch, 1841) has a generation time of 50–60 days).

For this reason, detailed study of mygalomorph development requires a model species whose embryos can be reliably collected in the field, occur in large clutches, and develop synchronously. To aid the study of mygalomorph development, we provide below information on one promising theraphosid species that could serve as a useful representative of Mygalomorphae in comparative developmental biology.

Aphonopelma hentzi (Girard, 1852) (Fig. 1) is of particular interest for comparative developmental studies, due to its broad geographic range, non-threatened conservation status, and accessibility of collection sites. This species has the widest distribution of any theraphosid spider in the United States and can be found in a variety of xeric habitats throughout portions of nine different states (Hamilton et al. 2016). Adult females are typically 12–13 cm in leg span and inhabit individual burrows for the duration of their 20–30 year lives (Baerg 1938; Hamilton et al. 2016). Upon maturity, males depart their burrows and seek mates. In southeastern Colorado, males are commonly encountered on rural roadways during the fall but generally do not survive past November (Hendrixson, pers. obvs.). Females that have successfully mated will overwinter in plugged burrows and emerge to feed the following spring while preparing to produce egg sacs (Hendrixson pers. obvs.).

METHODS

Field collection during the reproductive season.—In July of 2016, a short expedition to the Comanche National Grasslands near La Junta, Colorado (elevation 1353 m), was undertaken during the 2016 International Congress of Arachnology. Our field site, located near the northern limits of the distribution for this species (Hamilton et al. 2016), can be characterized as typical shortgrass prairie and rangeland. During this excursion, approximately 20 *A. hentzi* burrows were found; of these, three adult females were observed holding egg sacs with their pedipalps near the mouth of the burrow during the warmest part of the day (1000 to 1300 hr). The search was conducted over approximately five hours, with a search party of five persons.

The burrows are immediately recognizable by their circular shape (3–5 cm in diameter) and characteristic silk cover (Fig. 1A); *A. hentzi* does not use a trapdoor similar to that observed in many other mygalomorph spiders. If the silk cover is removed or damaged (Fig. 1B), the inhabitant will often begin repairing the cover after a few minutes, provided observers do not disturb the animal with sudden movements. Other spiders

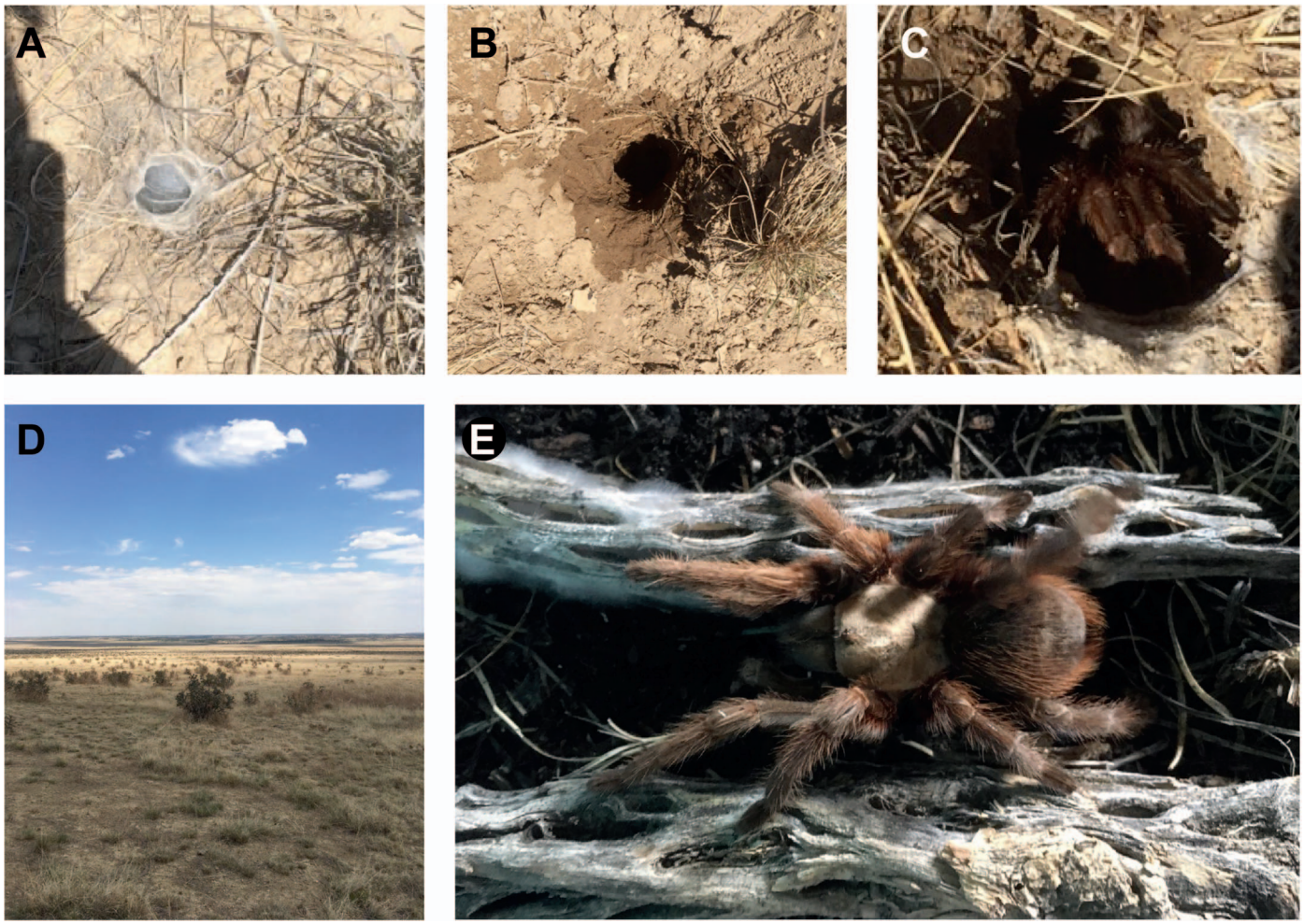

Figure 1.—Field collection of the mygalomorph *Aphonopelma hentzi*. Overview of field site at the Comanche National Grasslands, La Junta, CO. (A) Burrow of a *A. hentzi* juvenile (ca. 2.5 cm in diameter), with an intact silk cover. (B) Same burrow shown in A with silk removed; dampness around the entrance is the result of flushing the burrow with water. (C) Adult *A. hentzi* female emerging from the burrow. (D) Overview of the field site with the highest density of *A. hentzi*. (E) Adult female resting atop dried wood (in captivity).

discovered at this field site (agelenids, lycosids, and theridiids) did not make comparable webs or burrows, facilitating the identification of *A. hentzi*. Burrows of this species are unpredictable in their trajectory, often taking tight turns, and the vertical distance from aperture to floor ranged up to 25 cm.

Looking inside the burrow entrance can reveal if an egg sac is present if the female is holding it near the entrance (presumably, for thermoregulation of the embryos). The egg sac appears as a large sac (ca. 2–4 cm diameter), white in background color and speckled with debris particles (Fig. 2F). Embryos procured during this search were either close to hatching (clear deposition of cuticle; eyes visible) or were already in post-embryonic stages (the "larval" stage of spider development).

A second collecting effort was conducted from 10–22 June 2018, targeting embryos of younger (i.e., early limb development) stages for gene expression assays. This time of year was chosen based on our previous field observations about the first appearance of egg sacs, the stage of the embryos collected from these egg sacs in 2016, and extrapolations from the comparative development of the laboratory model spiders *Cupiennius salei* (Keyserling, 1877) (Trechaleidae) (Wolff & Hilbrant 2011) and *Parasteatoda tepidariorum* (Mittmann & Wolff 2012).

Over 200 burrows were examined during this 10-day period. All specimens we encountered were juveniles of undetermined sex or adult females, as determined by lack of swollen distal ends of the palps or mating claspers on the tibiae of leg I. Burrows were flagged with neon tape to allow repeat visits.

Procurement of egg sacs versus post-embryonic individuals.—Two conventional techniques are typically used for extracting *Aphonopelma* from their burrows: digging the specimen out, following the trajectory of the burrow; and flushing the animals out of the burrow by pouring a gentle, steady stream of water into the opening to simulate flooding (Hendrixson et al. 2013). Wetting the underside of the opisthosoma proved especially effective in convincing the spider to leave its burrow (Figs. 1B–C). Intriguingly, we never observed females with egg sacs when using this method. Over eight days, we flushed over 100 burrows of adult females, but obtained no egg sacs using this strategy. In four cases, we observed that females clearly

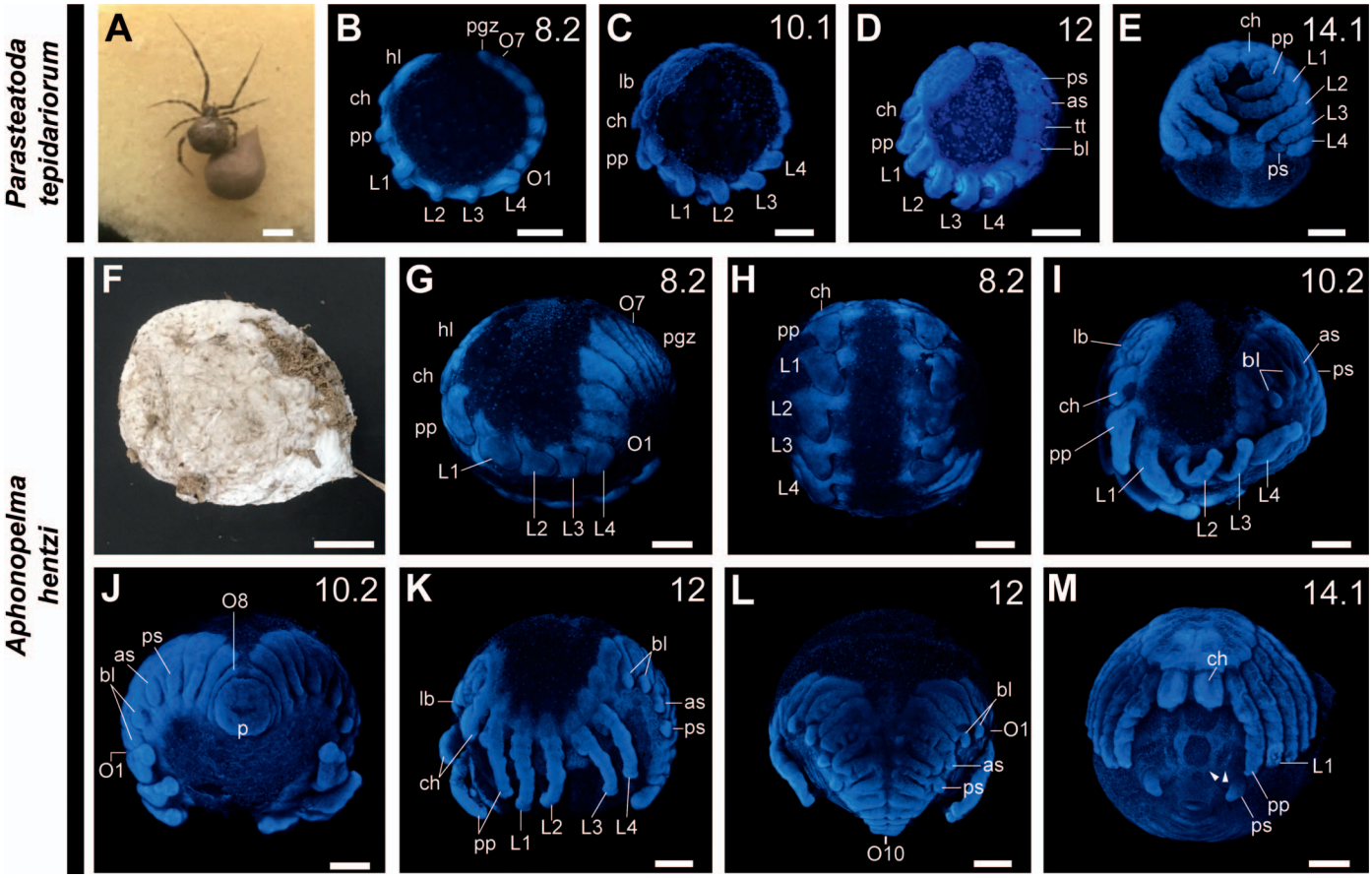

Figure 2.—Comparative development of the cobweb spider *Parasteatoda tepidariorum* (Entelegynae: Theridiidae) and *Aphonopelma hentzi*. (A) Intact egg sac of *P. tepidariorum* with female (in captivity). B–E: Embryogenesis of *P. tepidariorum*. Lateral views of the stages where anterior limb buds form (B), posterior limb buds form (C), appendages are fully elongated (D). The embryo of *P. tepidariorum* consistently maintains a "C" shape around the circumference of the spherical egg. After completion of inversion and dorsal closure (not shown), ventral closure occurs (E, in ventral view) and the embryo takes on the typical spider morphology; note the location and relative size of the spinnerets relative to the legs. F–M: Development of *A. hentzi*. (F) A typical egg sac of *A. hentzi*. (G) Lateral view of a stage where limb buds have formed; note the inflection of the opisthosoma at this stage (compare to B). (H) Same embryo as in G, in ventral view. (I) Lateral view of an older stage where the prosomal appendages are elongated, the opisthosomal limb buds have formed, and brain differentiation is ongoing (compare to C). (J) Same embryo as in I in posterior view, showing the limb buds of the opisthosoma (dorsal is to the left); note that at this stage, only eight opisthosomal segments have been formed. (K) Lateral view of *A. hentzi* at a later stage in development (compare the location of the embryo's posterior terminus to its counterpart in I). (L) Same embryo as in K in posterior view (dorsal is to the left); note that ten opisthosomal segments are now visible at this stage. (M) Upon completion of ventral closure, the embryo takes on the typical spider morphology. The central nervous system's ganglia are clearly visible on the ventral surface of the opisthosoma (white arrowheads). The anterior spinnerets have regressed and only the posterior spinnerets are visible. Scale bars in A and F are 1cm, 100 μm in B–E, and 250 μm in G–M. Anterior is to the left in panels B–D, G, H, I, and K; anterior is up in panels E and M. Abbreviations: as, anterior spinneret; bl, book lung; ch, chelicera; hl, head lobe; lb, labrum; L1, first walking leg; O1, first opisthosomal segment; p, proctodeum; pgz, posterior growth zone; pp, pedipalp; ps, posterior spinneret; tt, tubular tracheae.

holding egg sacs would retreat from sight when water was poured into the burrow (multiple trials per female, with repeated visits over three days); the female would then eventually leave the burrow without the egg sac ($n = 2$) or not leave the burrow at all ($n = 2$).

Instead, we found that egg sacs can be more effectively collected by removing the silk from the burrow cover and extracting the egg sac using a long (>20 cm) pair of forceps. Identification of females with egg sacs is greatly facilitated by a flashlight of over 700 lumens (particularly in the brightest part of the day), as some females with egg sacs may be found several centimeters below the burrow opening. For best results, forceps should be inserted with as little vibration as possible and the outer silk of the egg sac gripped securely. Extraction of the egg sac must be achieved by a quick and decisive set of motions, as females will retreat at once with the egg sac if it is disturbed. Using this approach, we collected eight egg sacs within 30 person-hours (two-person search party). This collection set comprised two egg sacs containing post embryonic stages, five egg sacs encompassing a range of limb bud stages, and one egg sac containing embryos at the germ disc stage. More egg sacs were found in the afternoon than in the morning; we postulate that this reflects the need to regulate the temperature of the embryos during the warmest part of the day (Supplementary Table 1, online at http://dx.doi.org/10.1636/JoA-S-18-081.S4).

Development of *A. hentzi* embryos in the laboratory.—Eight egg sacs of *A. hentzi* were returned from the field to the laboratory. Eggs are easily maintained in a six-well plastic plate with a matching lid, which permits wet paper towel to be placed in between the wells to maintain humidity. Eggs damaged during the process of collection and/or of opening the silk egg sac must be removed to prevent mold from infecting undamaged embryos. Embryos were developed at room temperature until reaching desired stages. Egg sacs contained 200–400 eggs and each egg measured approximately 2.4 mm in diameter (Fig. 2). The hatching rate in captivity for unhatched egg sacs exceeded 90% for embryos from both the 2016 and 2018 collections.

The stage of the embryo can be obscured by the chorion, which is granular in texture and light yellow in color under fluorescent light. Propitiously, the eggs of this species generally develop synchronously within each egg sac. To follow development, three to five embryos can be sampled and their stage examined by immersion in clove oil or halocarbon-700 oil, following Holm (1941).

Fixation of embryos.—Embryos were dechorionated using a 50% or 100% bleach solution (100% bleach = 8.25% sodium hypochlorite solution) until dissolution of the chorion enabled clear visualization of the embryo through the transparent vitelline membrane (3–5 min with gentle agitation; if visualization of development is desired, embryos will survive longer after 50% bleach solution washes). Embryos were subsequently washed with three rapid rinses of deionized water, followed by 3 × 5 min with 1X phosphate buffered saline (PBS). For fixations appropriate for gene expression surveys (Figs. 3A–B"; see Supplementary Files 1 and 2, online at http://dx.doi.org/10.1636/JoA-S-18-081.S1 and http://dx.doi.org/10.1636/JoA-S-18-081.S2), batches of ca. 40 embryos were washed into a 20 mL scintillation vial with 3.4 mL 1X PBS, 0.6 mL 37% formaldehyde, and 4 mL of heptane; rapid agitation of the vial resulted in the accumulation of embryos in the phase between the heptane and the formaldehyde/PBS solution. Fixation was conducted overnight (9–10 h) at 4°C with gentle agitation on a platform shaker. For fixations appropriate for protein expression surveys (Figs. 3C, D"; see Supplementary Files 1 and 3, online at http://dx.doi.org/10.1636/JoA-S-18-081.S1 and http://dx.doi.org/10.1636/JoA-S-18-081.S3), the same procedure was followed, but the fixation time was reduced to 7 h at 4°C. Fixations were stopped with several washes with 0.02% Tween-20 in 1X PBS (PBST), followed by gradual dehydration (>20 minutes) into 100% methanol for long term storage at -20°C (see Supplementary File 1, online at http://dx.doi.org/10.1636/JoA-S-18-081.S1).

Fluorescent immunohistochemistry.—Fixed embryos were gradually rehydrated into 0.05% Triton X-100 in 1X PBS (PBSTx) and washed 3 × 5 min with 1X PBSTx. Embryonic tissues were blocked for 1 h at room temperature (5% normal goat serum [ThermoFisher Scientific, USA] and 0.1% bovine serum albumin [Sigma Aldrich, USA] in 1X PBSTx) and incubated with primary antibodies for 3 days at 4°C. Primary antibodies consisted of the mitotic marker phospho-histone H3 (rabbit; PA517318 ThermoFisher) and the nervous system marker horseradish peroxidase (Alexa Fluor 647-conjugate; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) with a concentration of 1:200 for each antibody. Subsequent to several PBSTx washes (3 × 5 min; 3 × 10 min; 2 × 60 min), embryos were incubated in secondary goat anti rabbit antibody (TRITC; 1:200) for 24 h at 4°C. After a final set of PBSTx washes (3 × 5 min; 3 × 10 min; 2 × 60 min) embryos were incubated with a 1:2000 solution of Hoechst 33342 in 0.02% Tween-20 in 1X PBSTx for 30 min. For general embryonic morphology, embryos were only treated with the Hoechst wash in PBST, extended over 8-10 h at 4°C. Imaging was performed using a Zeiss 710 fluorescent confocal microscope driven by Zen software.

Gene cloning and *in situ* hybridization.—Protocols for RNA extraction, cDNA synthesis, transcriptomic sequencing, and library assembly follow our previously published protocols (Sharma et al. 2014; Setton et al. 2017). Description of the developmental transcriptome of *A. hentzi* is beyond the scope of this report and will be provided elsewhere (Setton & Sharma unpublished data). Homologs of the leg-patterning genes *dachshund-1* and *Sp6/9*, which have recently been examined in *P. tepidariorum* (Setton & Sharma 2018), were identified in the *A. hentzi* developmental transcriptome using best reciprocal BLAST searches, multiple sequence alignment, and maximum likelihood inference of the gene tree topologies. Gene-specific primers design (Supplementary Table 2, online at http://dx.doi.org/10.1636/JoA-S-18-081.S5), amplification, cloning, and synthesis of riboprobes was performed as previously described (Setton et al. 2017). Sequence data have been deposited in GenBank under accession numbers MK313693-MK313694.

Colorimetric *in situ* hybridization followed protocols for *P. tepidariorum* (Akiyama-Oda & Oda 2003, 2006; reagents as in Setton & Sharma 2018), with the following modifications: the post fix step on day one was 2 × 20 min, we extended the recommended length of the prehybridization step in day one to 3 hours, and the blocking step on day two was 2 × 30 minutes (Supplementary File 2, online at http://dx.doi.org/10.1636/JoA-S-18-081.S2).

Imaging was performed using a Nikon SMZ25 fluorescent stereomicroscope with 405 nm illumination and a DS-Fi2 digital color camera driven by Nikon Elements software. Staging of *A. hentzi* embryos was established using comparisons to the detailed and existing staging systems for the trechaleid *Cupiennius salei* (Wolff & Hilbrant 2011) and the theridiid *Parasteatoda tepidariorum* (Mittmann & Wolff 2012).

RESULTS AND DISCUSSION

Morphology and embryogenesis.—Aside from its greater size, the embryogenesis of *A. hentzi* embryos (Fig. 2) is notably different from that of *P. tepidariorum* in that the tail of the germ band curls downward in an increasingly pronounced manner during germ band elongation and inversion. This mode of opisthosomal development is comparable in haplogynes, mesotheles, Amblypygi, Uropygi, scorpions, and pseudoscorpions, in addition to other genera of mygalomorphs (Anderson 1973); only in derived araneomorphs (e.g., entelegynes) does the embryo maintain a C-shape around the circumference of a spherical egg (like in *P. tepidariorum*; Figs. 2B–D). This pattern of development with a downturned opisthosoma may reflect a homologous condition common to Arachnopulmonata (*sensu* Sharma et al. 2014) or the clade

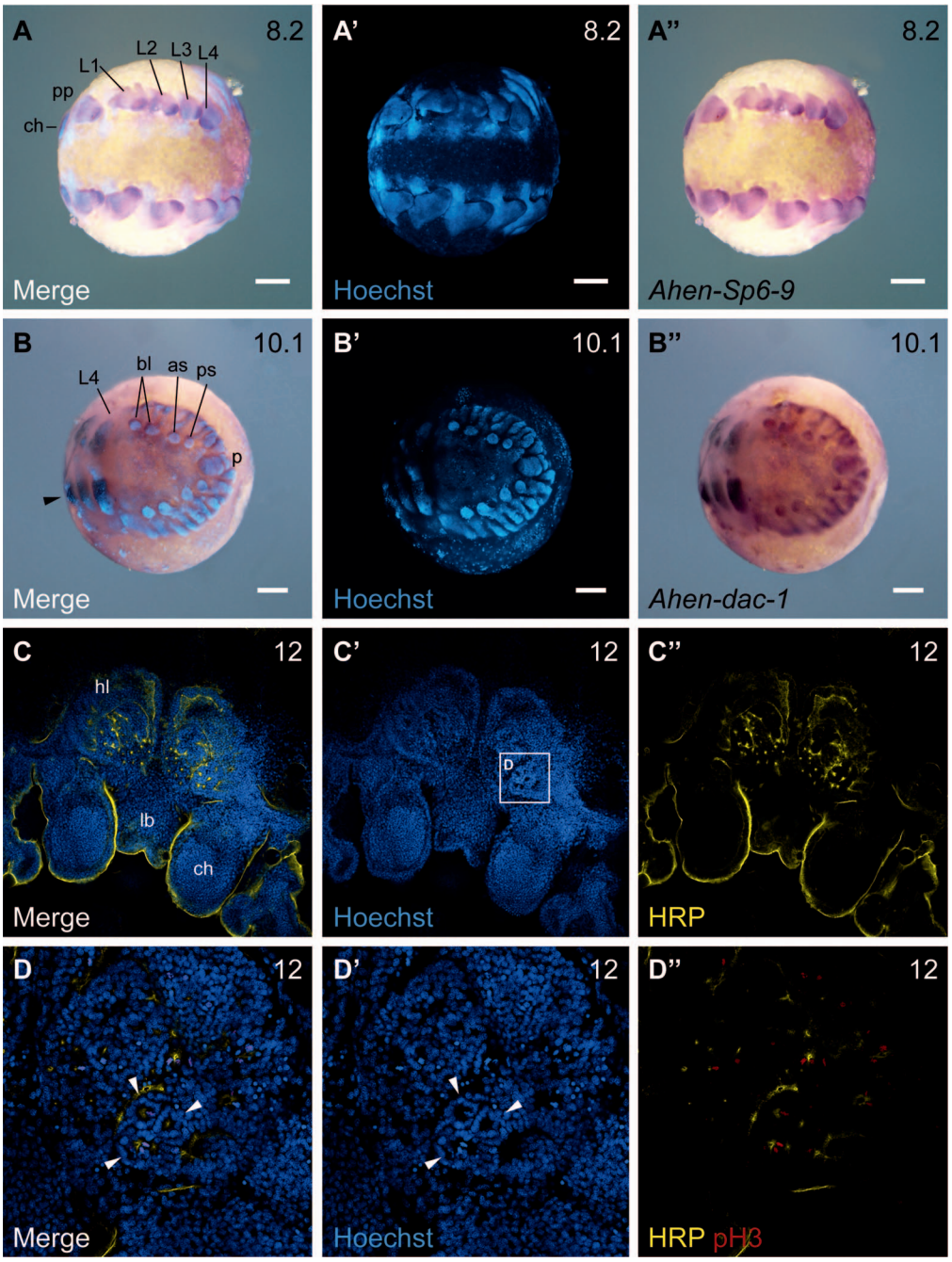

Figure 3.—Whole mount *in situ* hybridization and immunohistochemistry. A-A". Expression of the leg selector gene *Ahen-Sp6-9* in the prosoma of an *A. hentzi* embryo at stage 8.2. B-B". Expression of the leg gap gene *Ahen-dac-1* in the opisthosoma of *A. hentzi* at stage 10.1. Note the medial band of expression in the prosomal appendages (arrowhead). C-C" Central nervous system of the developing head at stage 12 visualized at 10X magnification with horseradish peroxidase primary antibody (HRP; yellow). The white box bounds the developing lateral eye and corresponds to the area magnified by image series D. D-D" Developing lateral eyes of the embryo in image series C at 20X with the central nervous system visualized using HRP (yellow) and cell division labeled with phosphorylated-histone H3 (pH3; red). "Merge" refers to the digital integration of multiple channels of the same embryo, resulting in a merged image. scale bars in A-B" are 250 μm, anterior is to the left in panels A-B", and dorsal is up in panels C-D". Abbreviations as in Figure 2.

Pseudoscorpiones + Arachnopulmonata (recovered in some phylogenomic analyses; Sharma et al. 2014, Ballesteros & Sharma 2019), with derivation of the C-shaped germband in Entelegynae (Turetzek & Prpic 2016) (Fig. 4). Furthermore, inversion—the ventral splitting of the germband with dorsal migration of the two halves of the embryo—is clearly a synapomorphy of all Tetrapulmonata, as it occurs in spiders and exemplars of Amblypygi and Thelyphonida (Anderson 1973; Weygoldt 1975) (embryonic development of Schizomida is entirely unknown).

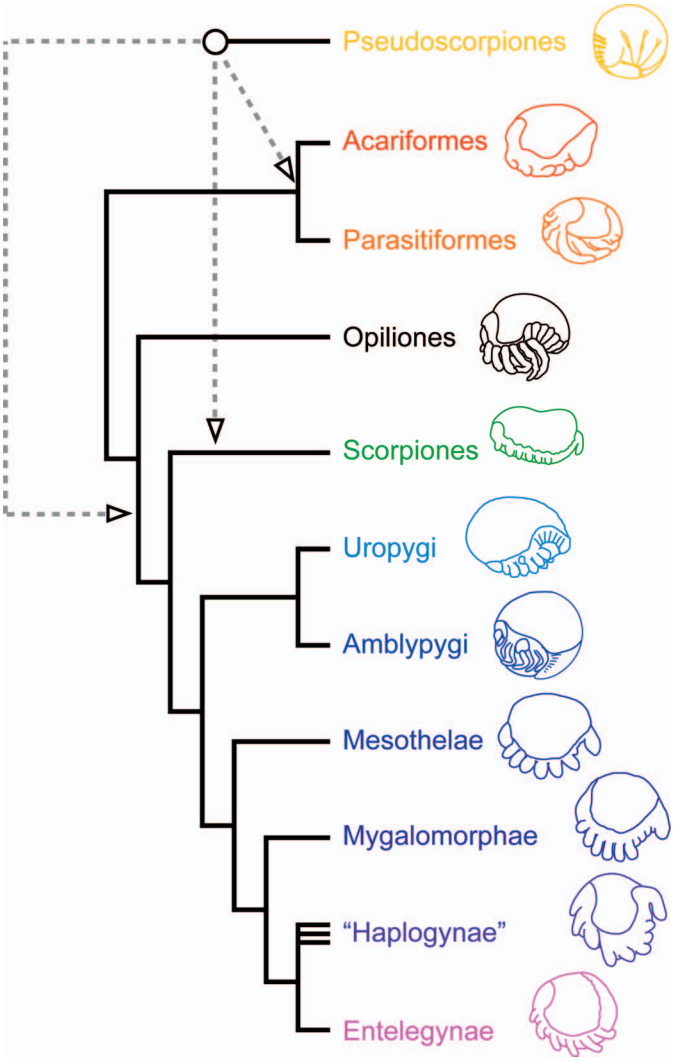

Figure 4.—Schematics of chelicerate embryos showing that the downturned opisthosoma is a plesiomorphic condition for Arachnopulmonata. The C-shaped germband in Entelegynae constitutes a derived state. Tree topology, particularly uncertainty about the placement of Pseudoscorpiones, reflects relationships recovered by Sharma et al. (2014) and Ballesteros & Sharma (2019). Line drawings are adapted from Weygoldt (1969, 1975), Anderson (1973), Farley (2001), Barnett & Thomas (2013) and Santos et al. (2013).

Due to the angle of inflection of the opisthosoma during inversion, the embryos of *A. hentzi* have clearly observable limb buds corresponding to the book lungs (on the second and third opisthosomal segments) and the spinnerets (on the fourth and fifth opisthosomal segments). While postembryonic stages of theraphosids bear only posterior median and posterior lateral spinnerets (Galiano 1996), the mygalomorph embryo has limb buds corresponding to both the anterior spinnerets (O4) and posterior lateral spinnerets (O5). Posterior median spinnerets are not visible during the embryonic stages we observed and were first seen at hatching; these are more easily visualized in the first instar.

During embryonic development, the anterior and posterior spinneret buds are initially comparable in size and shape. At a later stage in development, the anterior lateral spinnerets are dwarfed by the length of the posterior lateral spinnerets, reflecting the condition found in typical mygalomorph adults (Fig. 2M). By hatching, the anterior spinnerets are not observable; the mechanism of their regression is not known. Notably, segmentation of the posterior lateral spinneret does not occur embryonically; the hatchling ("larva" or post-embryo stage) retains an unsegmented posterior spinneret after embryogenesis, through the post-embryonic stage. Segmentation of this spinneret first appears after the first molt; this observation is in accordance with those of previous studies in other mygalomorph species (e.g., Galiano 1996).

The youngest egg sac we procured contained embryos at the cumulus stage on the day of collection (equivalent to stage 5 of *P. tepidariorum*) and the oldest contained post-embryos (hatchlings) (see Supplementary Table 1, online at http://dx.doi.org/10.1636/JoA-S-18-081.S4). These observations of variance in developmental stage, as well as the occurrence of post-embryos in mid-July, is contrary to the conventional understanding that *A. hentzi* produces egg sacs in July. We extrapolate from the date that post-embryos were collected in June that those egg sacs must have been lain at least in May or possibly as early as April of 2018. The least synchronous egg sac procured contained embryos ranging from the equivalent of *P. tepidariorum* stage 7 to stage 12. It is unknown why non-synchronous egg sacs occur in this species, though we have consistently observed in the theridiid *P. tepidariorum* that old females, as well as females that were fed poorly during maturation, will produce similarly asynchronously developing embryos.

Gene expression assay and immunohistochemistry.—To demonstrate the tractability of *A. hentzi* for expression assays and verify the quality of our fixation protocol, we conducted both *in situ* hybridization and immunohistochemistry assays for various stages of *A. hentzi* development. We first investigated the expression pattern of *Sp6-9*, a leg selector gene whose homolog we recently studied in *P. tepidariorum* (Setton & Sharma 2018). We recovered an expression pattern consistent with that of embryos of *P. tepidariorum* at a similar developmental stage. Expression of *Ahen-Sp6-9* is restricted to strong expression in the distal portions of the developing prosomal limbs with a weaker band of expression in the proximal part of the prosomal appendages (Fig. 3A-A"). As with the *P. tepidariorum* homolog, we observed no expression of *Ahen-Sp6-9* in the book lung or spinneret primordia.

Another gene utilized for testing *in situ* hybridization protocols was the medial leg marker *dachshund-1*, which has a published expression pattern in the theraphosid *Acanthoscurria geniculata* (C.L. Koch, 1841) (Pechmann & Prpic 2009) and in multiple araneomorphs (e.g., Abzhanov & Kaufman 2000; Prpic et al. 2003; Setton & Sharma 2018). *Ahen-dac-1* is expressed comparably to its homologs in other spider species, with expression in the medial parts of the developing prosomal appendages, spinnerets, book lungs, and nervous system (Fig. 3B'-B"). Notably, the expression patterns of both genes were clearer when stock probe concentrations were weaker (i.e., 200 µg/µL stocks provided better signal to noise ratios than 400 µg/µL stocks) (see Supplementary File 2, online at http://dx.doi.org/10.1636/JoA-S-18-081.S2).

Immunohistochemistry protocols were performed on late stage embryos to visualize neurogenesis and cell division in the

developing head. Figure 3C-C" provides an overview of neurogenesis at stage 12 of development. The area in 3C' bounded by a white box corresponds to the developing lateral eyes. The points of HRP expression inside this area show invaginating cells in the developing lateral eye field (arrowheads), and are magnified in Figure 3D-D". Here we show that the lateral eye field is not only undergoing key neurogenic events at this stage, but also pronounced mitotic activity, as visualized by the pH3 primary antibody. This is consistent with previous observations of neurogenesis and head development in other spider species (Stollewerk et al. 2001).

Several characteristics of *A. hentzi* are conducive to the establishment of this species as a model system for study of mygalomorph development, such as its non-threatened conservation status, large broods, ease of embryo and adult female maintenance in captivity, and ease of collection. Embryos are readily reared in the laboratory and are amenable to gene expression assays and immunohistochemistry. Additionally, our observations from the field suggest that egg sacs may be deposited earlier in the year than previously thought; obtaining embryos at the earliest stages of development may require field collection in late April to early May within the Colorado range of *A. hentzi*.

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