Appendage abnormalities in spiders induced by an alternating temperature protocol in the context of recent advances in molecular spider embryology

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In the literature there are abundant reports on developmental deformities in arthropods collected in their natural habitat. Since such teratogenically affected individuals are found purely by chance, the causes of their defects are unknown. Numerous potential physical, mechanical, chemical, and biological teratogens have been considered and tested in the laboratory. Thermal shocks, frequently used in teratological research on the spider *Eratigena atrica*, have led to deformities on both the prosoma and the opisthosoma. In the 2020/2021 breeding season, by applying alternating temperatures (14°C and 32°C, changed every 12 hours) for the first 10 days of embryonic development, we obtained 212 postembryos (out of 3,007) with the following anomalies: oligomely, heterosymely, bicephaly, schistomely, symely, polymely, complex anomalies, and others. From these we selected six spiders with defects on the prosoma and two with short appendages on the pedicel for further consideration. The latter cases seem particularly interesting because appendages do not normally develop on this body part, viewed as the first segment of the opisthosoma, and appear to represent examples of atavism. In view of the ongoing development of molecular techniques and recent research on developmental mechanisms in spiders, we believe the observed phenotypes may result from the erroneous suppression or expression of segmentation or appendage patterning genes. We consider “knockdown” experiments described in the literature as a means for generating hypotheses about the sources of temperature-induced body abnormalities in *E. atrica*.

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Short title: Appendage abnormalities in spiders

Abstract

In the literature there are abundant reports on developmental deformities in arthropods collected in their natural habitat. Since such teratogenically affected individuals are found purely by chance, the causes of their defects are unknown. Numerous potential physical, mechanical, chemical, and biological teratogens have been considered and tested in the laboratory. Thermal shocks, frequently used in teratological research on the spider *Eratigena atrica*, have led to deformities on both the prosoma and the opisthosoma. In the 2020/2021 breeding season, by applying alternating temperatures (14°C and 32°C, changed every 12 hours) for the first 10 days of embryonic development, we obtained 212 postembryos (out of 3,007) with the following anomalies: oligomely, heterosymely, bicephaly, schistomely, symely, polymely, complex anomalies, and others. From these we selected six spiders with defects on the prosoma and two with short appendages on the pedicel for further consideration. The latter cases seem particularly interesting because appendages do not normally develop on this body part, viewed as the first segment of the opisthosoma, and appear to represent examples of atavism. In view of the ongoing development of molecular techniques and recent research on developmental mechanisms in spiders, we believe the observed phenotypes may result from the erroneous suppression or expression of segmentation or appendage patterning genes. We consider "knockdown" experiments described in the literature as a means for generating hypotheses about the sources of temperature-induced body abnormalities in *E. atrica*.

Keywords: Developmental anomalies, Spider embryogenesis, Temperature fluctuations, Teratology, Thermally disturbed embryogenesis

42

43 Introduction

44 In natural aquatic and terrestrial habitats animals with body deformities are relatively common.
45 This observation applies particularly to arthropods, including crustaceans, insects, myriapods,
46 and chelicerates (e.g., *Estrada-Peña, 2001*; *Asiain & Márquez, 2009*; *Leśniewska et al., 2009*;
47 *Fernandez, Gregati & Bichuette, 2011*; *Feuillassier et al., 2012*; *Kozel & Novak, 2013*; *Scholtz,*
48 *Ng & Moore, 2014*; *Di, Edgecombe & Sharma, 2018; Levesque et al., 2018*; *Brenneis & Scholtz,*
49 *2021*). Since malformed arthropods are found purely by chance—e.g., during field research—the
50 causes of their abnormalities remain unknown. Various hypotheses have attempted to explain the
51 origin of these defects, sometimes affecting only one body part or organ, with a variety of
52 physical, mechanical, chemical, and biological factors proposed (e.g., *Miličić, Pavković-Lučić &*
53 *Lučić, 2013*).

54 Potential teratogenic factors can be tested in laboratory experiments using invertebrates,
55 including species considered models for study (*Lagadic & Caquet, 1998*). A number of chemical
56 reagents (e.g., *Ehn, 1963a*; *Ehn, 1963b*; *Itow & Sekiguchi, 1980*; *Köhler et al., 2005*; *Pinsino et*
57 *al., 2010*), radiation (*Seitz, 1966*; *Seitz,1970*; *Yoshikura, 1969*; *Matranga et al., 2010*), high
58 humidity (*Buczek, 2000*), low/high temperature (*Napiórkowska & Templin, 2012*), and
59 mechanical disturbance/manipulation (*Holm, 1952*; *Sekiguchi, 1957*; *Scholtz & Brenneis, 2016*)
60 have already been exploited in teratology research. For instance, *Holm (1940)* hypothesized a
61 teratogenic effect of temperature on spiders and later *Juberthie* (e.g., *1968*) investigated the
62 effect of supraoptimal temperature on embryogenesis in harvestmen (Opiliones). Subsequently,
63 the research was extended by using abrupt temperature changes during the incubation of

Eratigena (formerly *Tegenaria*) *atrica* (C.L. Koch, 1843) embryos (e.g., *Jacuński, 1984*; *Jacuński & Templin, 2003*; *Napiórkowska, Jacuński & Templin, 2010a*; *Napiórkowska, Jacuński & Templin,2010b*; *Napiórkowska, Napiórkowski & Templin, 2016a*; *Napiórkowska, Napiórkowski & Templin, 2016b*; *Napiórkowska, Templin & Napiórkowski, 2021*). It was observed that the application of alternating temperatures (lower and higher than the optimum) during early embryogenesis could lead to a range of deformities in both body tagmata. The most severe defects led to high embryo mortality or made it difficult for embryos to hatch to the postembryo stage. Moreover, some hatched but deformed individuals were unable to lead a normal life and achieve reproductive success. Anomalies described in *E. atrica* have included: oligomely (absence of one or more appendages), symely (fusion of contralateral appendages), schistomely (bifurcation of appendages), heterosymely (fusion of ipsilateral appendages), polymely (presence of one or more additional appendages), bicephaly (partial prosomal duplication), and so-called complex anomalies (two or more categories of anomaly occurring simultaneously) (e.g., *Jacuński & Napiórkowska, 2000*; *Jacuński et al., 2002*; *Jacuński, Templin & Napiórkowska, 2005*; *Napiórkowska, Jacuński & Templin, 2007*; *Napiórkowska & Templin, 2012*; *Napiórkowska & Templin, 2013*; *Napiórkowska, Templin & Napiórkowski, 2013*; *Napiórkowska et al., 2016; Napiórkowska, Templin & Wołczuk, 2017*).

At the Nicolaus Copernicus University in Toruń, Poland, teratological research on spiders by application of a thermal factor has been carried out since the 1970s (e.g., *Mikulska, 1973*). Since then, various anomalies have been described, with new cases recorded every year. Although early studies focused mainly on morphological description of teratologically altered individuals, attempts to explain the causes of deformities were also made. *Jacuński (1984; 2002)* suggested that many induced anomalies seen in late embryos or postembryos were presaged by structural

aberrations evident in embryos as early as the blastoderm stage. For instance, thermal shocks led to the appearance of gaps in the blastoderm that *Jacuński* (*1984*) proposed may eliminate some embryo fragments, causing oligomely. On the other hand, it was suggested that symely or heterosymely could result if thermal treatment brought parts of the blastoderm closer together than was normal. However, *Jacuński* (*2002*) also noted that anomalies appearing well into embryonic development, e.g., during the limb bud elongation stage (see *Wolff & Hilbrant, 2011*), were not necessarily preceded by obvious structural abnormalities in earlier stages. This demonstrates that interpreting teratologies only in mechanistic terms is at best insufficient.

Over the last quarter-century, molecular techniques have been enlisted for the functional analysis of genes involved in the development of body segmentation and appendage formation in spiders. In particular, extensive use of *in situ* hybridization, RNA interference (RNAi), and immunolabeling have provided much insight into the expression of many developmental regulatory genes during spider embryogenesis (*Damen & Tautz, 1998; Popadić et al., 1998*; *Abzhanov, Popadić & Kaufman, 1999*; *Schoppmeier & Damen, 2001*; *Stollewerk, Weller & Tautz, 2001*; *Stollewerk, Schoppmeier & Damen, 2003*; *Akiyama-Oda & Oda, 2003*; *Davis, D'Alessio & Patel, 2005*; *McGregor et al., 2008*; *Prpic, Schoppmeier & Damen, 2009*; *Schwager et al., 2009*; *Pechmann et al., 2011*; *Schwager, Meng & Extavour, 2015*; *Benton et al., 2016*; *Schwager et al., 2017*; *Oda & Akiyama-Oda, 2019*; *Heingård & Janssen, 2020*; *Setton & Sharma, 2021*; to cite but a few such studies). This work has demonstrated various spider anomalies that result from the suppression or misexpression of specific segmentation and appendage patterning genes, raising the expectation that at least some abnormalities induced by thermal shock to embryos will be explicable in these terms. Of course, this does not preclude the possibility of mechanisms less directly related to gene expression, but also affected by

110 temperature (e.g., changes in metabolism, abnormal cell movements), also or alternatively being
111 involved in creating defects.

112 In the 2020/2021 breeding season, using alternating temperatures during early embryogenesis of
113 *E. atrica*, we obtained 212 postembryos with various body deformities. Since many of these
114 anomalies have already been described in our previous works, we focused on those observed for
115 the first time or those particularly relevant to evo-devo research, such as the rare cases where an
116 appendage is found on the pedicel (petiolus, petiole) that connects the prosoma to the
117 opisthosoma. Regarding the latter, appendages on one or both sides of the pedicel in
118 postembryos of *E. atrica* were first described by *Jacuński* (*1971*; *1984*). Some postembryos so
119 afflicted did not survive beyond this stage, but for those that did, differences in the longevity of
120 these appendages were later noted in *Jacuński & Templin* (*1991*): in one individual the
121 appendage disappeared after the postembryo molted, while in another, a short, two-podomere
122 appendage was present until the 6th stadium. *Jacuński & Templin* (*1991*) additionally described a
123 postembryo of *E. atrica* with one substantial limb on the pedicel. Initially composed of four
124 podomeres, by the 5th stadium it resembled, in form and segmentation, a complete walking leg,
125 albeit distorted. During the 6th molt the leg broke off at the trochanter-femur joint. It grew back
126 starting with the 7th molt, but the spider died during the 9th molt when loss of the leg re-occurred,
127 accompanied by substantial loss of hemolymph. *Jacuński & Templin* (*1991*) proposed that the
128 presence of an appendage on the pedicel is an atavistic trait. They also questioned whether the
129 pedicel in spiders is correctly considered the first segment of the opisthosoma, since it has the
130 potential to develop appendages similar in size and structure to walking legs.

131 Our study was aimed at further exploring the diversity of developmental anomalies in
132 postembryonic *E. atrica* as induced by the alternating temperature protocol. We also sought to

begin interpreting abnormalities like those seen in the 2020/2021 breeding season, which included two rare cases of appendages on the pedicel, in terms of potential errors in developmental gene expression. For the latter we reviewed the literature related to the expression of such genes with a focus on functional studies performed on spiders that employed RNAi to knock down specific genes. Note that aberrant gene expression is broadly construed here to include abnormal expression resulting from such temperature-influenced effects as atypical cell movements, cell division, cell death, and changes in metabolism. Overall expression of a gene could thus be quantitatively normal potentially but still present as abnormal phenotypes if positional or temporal perturbations to expression deviate significantly from normal.

Material and methods

Teratological experiments on embryos of the spider *Eratigena atrica* (C.L. Koch, 1843) were carried out in the 2020/2021 breeding season. In September 2020, 32 sexually mature females and 24 males were collected from the vicinity of Toruń, Włocławek, and Chełmża, Poland. In the laboratory each individual was placed in a 250 cm^3 well-ventilated glass container, kept in a darkened room. A temperature of 21°C and a relative humidity (RH) of about 70% were maintained in the room throughout the experiment. Spiders were fed *Tenebrio molitor* larvae twice a week and water was supplied in soaked cotton balls. After three weeks, a male was introduced to each female for insemination. This procedure was repeated several days later with a different male to help ensure that all females were inseminated. First egg sacs were laid after a few weeks, followed periodically by additional egg sacs, averaging 7 or 8 egg sacs per female (in two previous breeding seasons) with up to 19 egg sacs constructed by a single female. All egg sacs were immediately removed from the containers and cut open to remove eggs, which were then counted and evenly divided into two groups: an experimental group and a control group. To

verify that most eggs were fertilized, three randomly selected eggs per egg sac were immersed in paraffin oil and inspected.

Embryos from the control group were incubated at a temperature of 22°C and 70% RH until hatching to the postembryo occurred, while embryos from the experimental group were exposed to alternating temperatures of 14°C and 32°C. The temperature was changed every 12 hours for 10 days, until segments of the prosoma appeared on the germ band and limb buds appeared on these segments [comparable to Stage 9 in the trechaleid *Cupiennius salei* (Keyserling, 1877) (*Wolff & Hilbrant, 2011*); Stage 8.2 in the theridiid *Parasteatoda* (formerly *Achaearanea*) *tepidariorum* (C.L. Koch, 1841) (*Mittmann & Wolff, 2012*)]. Subsequently, incubation was continued using the same conditions applied to the control group. After hatching, postembryos from both groups were examined for abnormalities in the prosoma and opisthosoma. Deformed individuals were photographed using a Zeiss Axiocam 105 color CMOS camera mounted on a Zeiss Axio Lab A1 light microscope and operated with Zen software (Version 2.3, blue edition).

We gathered references that present results of spider RNAi experiments, as they might shed light on potential gene misexpression leading to appendage abnormalities, as induced by the alternating temperature protocol.

Results

In the 2020/2021 breeding season, we obtained approximately 10,000 eggs/embryos, half of which constituted the control group. In this group, no hatched individuals with developmental defects were found, all postembryos having a properly developed prosoma with appendages and an opisthosoma with no observed abnormalities (Fig. 1). Approximately 10% of these controls failed to hatch, though development proceeded far enough in some that their fertilized status was

178 apparent. The remainder, however, no doubt included some unfertilized eggs, with a comparable
179 number presumably present in the experimental group, though we do not know what this number
180 was. Eggs (100 or more) in the first egg sac built by a female are usually all fertilized or nearly
181 so, but in subsequent egg sacs, which contain fewer total eggs, there are typically higher
182 percentages of unfertilized eggs.

183 In the experimental group embryo mortality was much higher. About 40% of all embryos died at
184 various stages of development; some failed to hatch from their eggshells even though their
185 embryonic development appeared complete. In total, 3,007 postembryos were obtained. Among
186 these, individuals with a normally developed body structure predominated (2,795; 93%). The
187 remaining postembryos (212) had various defects, most of which affected the prosoma and its
188 appendages, although in nine individuals (4% of abnormal postembryos) deformities were also
189 found in the opisthosoma. Oligomely was, by far, the most frequent anomaly, but multiple
190 examples of each of several other types of anomaly—heterosymely, bicephaly, schistomely,
191 symely, and polymely—were also obtained (Table 1). Moreover, >30% of postembryos
192 displaying abnormal phenotypes did not fall neatly into one of these five types. They included
193 individuals with complex anomalies, i.e., with multiple defects of more than one type, and those
194 with abnormalities not conforming to any of these five types, grouped in Table 1 as 'Other
195 abnormalities'. The latter group included postembryos with significantly shortened or deformed
196 appendages. Since many of the observed deformities have already been described in our previous
197 studies, we present only selected cases, either recorded for the first time (Fig. 2) or of two
198 postembryos with a short appendage on the pedicel (Fig. 3), constituting the only instances of
199 polymely observed during this breeding season (Table 1).

The complex anomaly in the spider in Fig. 2A affected only the right side of the prosoma while the left side was formed normally with six well-developed, segmented appendages: chelicera, pedipalp, and four walking legs (L1-L4). On the right side of the prosoma the chelicera was missing (oligomely) and two appendages emerged from the gnathocoxa (= gnathendite = gnathobase = endite = maxilla), a normal pedipalp and a short protuberance (labeled ‘a’ in Fig. 2A) that lacked segmentation and moved independently. The legs were normally developed. The spider in Fig. 2B was likewise affected by a complex anomaly on the right side of the prosoma only. The chelicera was represented by a small, mobile protuberance (labeled ‘a’ in Fig. 2B) and the pedipalp was absent (oligomely). The legs had a normal structure. The spider in Fig. 2C was also affected by oligomely, the deformity most frequently observed in the teratological material. On the right side of the prosoma this individual had a well-developed chelicera and pedipalp, but only three legs. On the left side of the prosoma there was a complete set of appendages. Bilateral oligomely, though less common, was also observed in the teratological material. This anomaly affected the spider shown in Fig. 2D. On the right side of the prosoma there were five appendages—a chelicera and four legs—with the pedipalp missing. On the left side of the prosoma there were also only five appendages—a chelicera, pedipalp, and three legs; one leg was missing. The individual in Fig. 2E was affected by schistomely of leg L2 on the right side of the prosoma. The bifurcation started in the middle of the metatarsus and included the tarsus. The schistomely was symmetric in that the two distal ends (‘a’ and ‘b’ in Fig. 2E) were about the same length. The remaining appendages, including chelicerae and pedipalps, showed no irregularities. The spider in Fig. 2F had an especially unusual anomaly that affected leg L4 on the left side of the prosoma, presenting as a slightly widened coxa from which only two short branches (‘a’ and ‘b’ in Fig. 2F) projected. The only visible segmentation on these branches was

a single articulation, possibly demarcating the trochanter. This anomaly may represent schistomely initiated proximally within the developing leg, forestalling much further development. On the right side all appendages were well developed.

Figures 3A and 3B present a rare anomaly. These two postembryos had a very short appendage on the pedicel that connects the prosoma and opisthosoma. This additional appendage was on the left side of the pedicel. In both cases no other abnormalities were apparent. In the spider shown in Fig. 3A, the shortened appendage had the thickness of a walking leg, but it was not segmented. It had two small, rounded protrusions located prolaterally and distally ('a' and 'b' in Fig. 3A). In the spider in Fig. 3B, the appendage on the pedicel was of similar length and (proximally) width to that on the other specimen, and it was segmented to the extent that the first podomere (coxa) could be distinguished. The appendage widened distally, ending in an uneven surface with several bumps.

Discussion

Using the established thermal method for inducing developmental abnormalities in spider embryos (*Jacuński, 1984*), we obtained 212 individuals with body defects in the 2020/2021 breeding season, representing 7% (212/3007) of the successfully hatched postembryos, and about 4% (212/5000) of the embryos (hatched and unhatched), in the experimental group. These fairly low percentages suggest that spiders, as ectotherms, possess mechanisms that help make them relatively resistant to sudden temperature changes. One such mechanism likely includes the expression of heat shock protein (*Hsp*) genes, encoding protein-folding chaperones. It has been shown that the expression of *Hsp* genes significantly increases in response to various environmental stressors, including high temperature (*Martínez-Paz et al., 2014* and references

245 therein). Other mechanisms are presumably also involved as some induced morphological
246 aberrations can be successfully eliminated by embryonic self-regulation and regeneration
247 processes (*Jacuński, 2002*; *Foelix, 2011*; *Oda et al., 2020*; *Oda & Akiyama-Oda, 2020*;
248 references therein). But the high mortality among experimental embryos (40%) as compared to
249 control embryos (10%) also suggests a relatively high percentage of induced abnormality in the
250 experimental group, severe enough to prevent hatching. It therefore appears that the alternating
251 temperature protocol was effective in disrupting normal development in about one-third of
252 embryos, causing a range of developmental anomalies and high embryo mortality. This thesis is
253 supported by an absence of developmental defects and low embryo mortality within the control
254 group.

255 We have noted a trend for mortality percentages in both control and experimental groups to rise
256 over the past decade (*Napiórkowska, Templin & Napiórkowski, 2013*; *Napiórkowska,*
257 *Napiórkowski & Templin, 2016a*; *Napiórkowska et al., 2016*; *Napiórkowska & Templin, 2017*;
258 *Napiórkowska, Templin & Napiórkowski, 2021*), from a low of 4% and 20%, respectively
259 (*Napiórkowska, Templin & Napiórkowski, 2013*), to the present study's high (10%, 40%,
260 respectively). Conversely, the percentage of successfully hatched postembryos in the
261 experimental group that exhibited defects has shown a downward trend over the same period,
262 from highs of 17-18% (*Napiórkowska, Templin & Napiórkowski, 2013*; *Napiórkowska et al.,*
263 *2016*) to a low of about 4% (*Napiórkowska, Templin & Napiórkowski, 2021*), rebounding
264 moderately in the present study with 7%. These opposite trends in the experimental group could
265 be related: if a larger percentage of embryos adversely affected by the alternating temperature
266 protocol fail to hatch, a smaller percentage of defective individuals may remain among the
267 embryos that hatch successfully. As yet we have no explanations for these trends.

In the teratological material, oligomely was the most frequent anomaly by a large margin, accounting for about 55% of cases, and it was even more prevalent considering that oligomely was a component in some postembryos (e.g., Fig. 2A, B) categorized as having 'Complex anomalies' (Table 1). Other anomaly categories were observed much less frequently, which agrees with the results of previous studies. If we express percentages by considering only the six conspicuous single anomaly categories (i.e., discounting 'Complex anomalies' and 'Other abnormalities' categories) as they occurred on prosomata and pedicels, cases of oligomely accounted for 79.6% of defects in this study. This percentage, across five earlier studies (*Jacuński, 1984*; *Napiórkowska, Templin & Napiórkowski, 2013*; *Napiórkowska et al., 2016*; *Napiórkowska & Templin, 2017*; *Napiórkowska, Templin & Napiórkowski, 2021*), ranged from 73.5-84.8%. In contrast, percentages for the other five single anomaly categories were (given as % for this study followed by % range in the five earlier studies): heterosymely, 8.2%, 4.9-10.4%; schistomely, 4.1%, 2.2-9.9%; bicephaly, 4.8%, 0-6.5%; symely, 2.0%, 0-7.8%; polymely, 1.4%, 0-3.7%.

Oligomelic postembryos

Molecular embryological research has suggested alterations to normal gene expression that might account for some instances of appendage loss. Parental RNAi (pRNAi) studies, especially in *P. tepidariorum*, have revealed a range of abnormal phenotypes from knockdown of selected developmental genes (*Oda & Akiyama-Oda, 2020*), depending on the specific gene suppressed and also on the degree of suppression of a given gene within different embryos. These phenotypes can include embryos exhibiting oligomely, but co-occurring abnormalities sometimes prevent hatching, indicating that widespread down-regulation of the targeted genes does not account for oligomelic postembryos like those in Fig. 2A-D. For example, knockdown

of the Notch-signaling-pathway component *Delta* in *P. tepidariorum* (*Pt-Delta*) (*Oda et al., 2007*) or the spider gap gene *Pt-Sox21b.1* (*Paese et al., 2018*; *Baudouin-Gonzalez et al., 2021*) results in loss of leg-bearing segments but this is accompanied by loss of all opisthosomal segments. More localized suppression, however, comparable to that achieved by embryonic RNAi (eRNAi) (*Oda & Akiyama-Oda, 2020*), cannot be ruled out in oligomelic postembryos.

As an aside, conspicuously lethal consequences of gene downregulation, as occur with *Pt-Delta* and *Pt-Sox21b.1* pRNAi, are potentially relevant to the high mortality that was observed in experimental *E. atrica* embryos. Also lethal and relevant is embryonic development that, from a superficial perspective, proceeds essentially to completion without obvious defect, but the embryo nevertheless fails to hatch. Embryos like these were among the 40% of the experimental group that did not hatch. Perhaps significantly, a comparable phenotype has been observed with suppression of any of three transcription factors by pRNAi: *Pt-foxQ2*, *Pt-six3.1*, or *Pt-six3.2* (*Schacht, Schomburg & Bucher, 2020*).

More likely to be involved in appendage losses like those in Fig. 2 are genes that, when knocked down, result in oligomelic embryos able to survive hatching. Two such genes, expressed during early embryogenesis and concurrent with application of our thermal treatment, are the gap gene *hunchback* (*hb*) (*Schwager et al., 2009*) and *Distal-less* (*Dll*), an appendage patterning gene that also plays an earlier gap gene role in spiders (*Pechmann et al., 2011*). pRNAi of *hb* in *P. tepidariorum* (*Pt-hb*) yields postembryos missing the L2 leg pair or both L1 and L2 legs (*Schwager et al., 2009*), while, similarly, pRNAi of *Pt-Dll* produces postembryos lacking the L1 leg pair or both L1 and L2 legs (*Pechmann et al., 2011*; *Setton et al., 2017*; *Setton & Sharma, 2018*). These losses reflect loss of the segments on which the legs would have developed and it is only segments bearing walking legs that are so affected (*Schwager et al., 2009*; *Pechmann et al.,*

2011), reflecting the distinction between segmentation of the head region, with its chelicerae and pedipalps, and that of the thorax region, with its four pairs of legs (*Kanayama et al., 2011*). If this also applies to *E. atrica*, then abnormal suppression of *Ea-hb* would not contribute to oligomely involving chelicerae and pedipalps (Fig. 2A, B, and D), but it could be a factor in spiders with missing legs (Fig. 2C, D). The same can be said for *Ea-Dll* suppression during its early involvement with prosomal segmentation (its gap gene role) (*Pechmann et al., 2011*). Later suppression of *Dll* in limb buds (*Chen, Piel & Monteiro, 2016*), whether preceded by early *Dll* suppression (pRNAi; *Pechmann et al., 2011*) or not (eRNAi; *Schoppmeier & Damen, 2001*; *Pechmann et al., 2011*), results in truncated appendages but not in any additional appendage loss.

There are, however, two confounding considerations where potential abnormal *Ea-hb* or *Ea-Dll* expression is concerned: (1) Though *Schwager et al.* (*2009*) did note left-right leg reduction asymmetry in *Pt-hb* pRNAi embryos, leg losses resulting from prosomal segment losses have usually been symmetric (*Schwager et al., 2009*; *Pechmann et al., 2011*), whereas the alternating temperature treatment applied in this study has often yielded asymmetric (Fig. 2C, D), as well as symmetric (e.g., *Jacuński, Templin & Napiórkowska, 2005*), leg oligomely. (2) We have not been able to determine which legs specifically have been missing in oligomelic postembryos, even after examining leg neuromeres in histological sections (*Jacuński, Templin & Napiórkowska, 2005*; *Napiórkowska, Napiórkowski & Templin, 2016b*), and therefore we do not know if leg losses have been consistent with *Ea-hb* or early *Ea-Dll* suppression.

Regarding (1), any *Ea-hb* or *Ea-Dll* inhibition induced by our thermal treatment might be more localized and asymmetric than that often resulting from pRNAi. Indeed, unilaterally oligomelic *E. atrica* with corresponding unilateral losses of leg nerves and ganglia indicate that thermally-induced disturbances result in losses of hemisegments more often than of full segments

(*Jacuński, 1983*; *Jacuński, Templin & Napiórkowska, 2005*; *Napiórkowska, Napiórkowski & Templin, 2016b*). This is reminiscent of asymmetric prosomal appendage shortening that has been induced in *C. salei* by knockdown of *Cs-Dll* using eRNAi (*Schoppmeier & Damen, 2001*) and of seven-legged postembryos that occasionally resulted from *Pt-Dll* pRNAi, indicating loss of a single L1 hemisegment (*Setton et al., 2017*). *Schoppmeier & Damen* (*2001*) noted the median furrow (ventral sulcus) that divides the right and left halves of the embryonic germ band (*Foelix, 2011*; *Wolff & Hilbrant, 2011*), and the seemingly independent development of the two halves, as a possible explanation for such asymmetric phenotypes.

Regarding (2), future studies could explore a strategy used by *Pechmann et al.* (*2011*) for ascertaining the identity of missing legs: for oligomelic postembryos able to molt successfully to at least 1st instars, the number and arrangement of slit sense organs on the sternum, compared to control spiders, should help identify the missing legs and provide an alternative to histological sectioning for indicating if symmetric/asymmetric oligomely of legs is accompanied by loss of an entire segment/hemisegment, as previously suggested based on histology (*Jacuński, 1983*; *Jacuński, Templin & Napiórkowska, 2005*; *Napiórkowska, Napiórkowski & Templin, 2015*; *Napiórkowska, Napiórkowski & Templin, 2016b*).

We should also note that, unlike RNAi experiments, in which the gene targeted by treatment is known, genes most directly impacted by application of the alternating temperature protocol may be cofactors, upstream regulators, or downstream targets of genes discussed here as being potentially perturbed by the protocol, rather than directly affecting expression of the candidate gene itself. For example, pRNAi of the transcription factor *Sp6-9* in *P. tepidariorum* (*Pt-Sp6-9*) has been observed to reduce or eliminate *Pt-Dll* expression (*Königsmann et al., 2017*; *Setton & Sharma, 2018*) as well as eliminate expression of the segment polarity gene *Pt-engrailed-1* (*Pt-*

en-1) in the L1 and L2 segments (*Setton & Sharma, 2018*), similar to the effect of *Pt-Dll* pRNAi on *Pt-en-1* expression (*Pechmann et al., 2011*). Resulting phenotypes include embryos missing these two segments and, so, the legs that would form on them (*Königsmann et al., 2017*; *Setton & Sharma, 2018*). Thus, thermally-induced defects consistent with inhibited *Ea-Dll* expression might actually reflect initial direct disruptions to *Ea-Sp6-9* expression. Also, genes most directly affected may vary among embryos depending on, e.g., exact timing of a temperature switch in relation to an embryo's stage of development.

On first consideration, missing pedipalps, as in Fig. 2B, D, could suggest disturbance to the normal expression of the Hox gene *labial* (*lab*), specifically the paralog *lab-1* (*lab-B* in *Schwager et al., 2017*), first expressed at Stage 4 in *P. tepidariorum* (*Pechmann et al., 2015*). Its knockdown by pRNAi can result in postembryos lacking pedipalps, though, unlike leg losses that are due to loss of the corresponding prosomal segments, the pedipalpal segment is retained (*Pechmann et al., 2015*). On the other hand, like the above pRNAi-induced leg losses, pedipalp loss as seen in *Pt-lab-1* pRNAi postembryos has been symmetric (*Pechmann et al., 2015*), whereas the alternating temperature treatment more often results in asymmetric pedipalp oligomely in *E. atrica* (Fig. 2B, D), suggesting a potential localized disruption to *Ea-lab-1* expression. However, an abnormal postembryo like that shown in Fig. 2B, in which the site of a missing pedipalp is adjacent to a greatly reduced chelicera (labeled 'a'), does not support this suggestion if we assume a shared genetic cause for both anomalies (this assumption is by no means certain). This is because expression of *lab-1* (or any of the Hox genes) is not involved in specifying chelicera morphology (*Pechmann et al., 2010*).

An alternative explanation that might encompass both defects has not yet emerged from functional studies in spiders. The gene *dachshund-2* is expressed proximally in both chelicerae

and pedipalps but the only noted phenotypic consequences of its knockdown by pRNAi in *P. tepidariorum* are malformed patellae in the walking legs (*Turetzek et al., 2015*). Two paralogs of *extradenticle* (*exd-1*, *exd-2*) and *homothorax-1* (*hth-1*) are also expressed proximally in pedipalps and chelicerae (*Prpic & Damen 2004*; *Pechmann & Prpic, 2009*; *Turetzek et al., 2017*) but *exd* has not been the subject of RNAi experiments in spiders, or any chelicerates (*Nolan, Santibáñez-López & Sharma, 2020*), and among chelicerates *hth* function has only been examined by eRNAi in the harvestman *Phalangium opilio* Linnaeus, 1758 (*Sharma et al., 2015*). However, studies in insects and spiders indicate that *exd-1* and *hth-1* of spiders are functionally linked (Hth-1 required for translocation of Exd-1 into the nucleus), such that knockdown of either gene would likely produce similar, though not identical, phenotypes (*Sharma et al., 2015*; *Turetzek et al., 2017*; references therein). Phenotypes resulting from knockdown of the single-copy *hth* in *P. opilio* (*Po-hth*) included homeotic transformations of chelicerae and pedipalps to leg identities, appendage truncation, and fusions between chelicerae and pedipalps, though, importantly, apparently not pedipalp oligomely (the Results do, however, state "The labrum and/or some appendages also failed to form" [among Class I phenotype embryos] (*Sharma et al., 2015*) without elaboration). Interestingly, like the aforementioned defect asymmetry observed in *Cs-Dll* eRNAi *C. salei* embryos (*Schoppmeier & Damen, 2001*), a high incidence of asymmetric defects was also obtained with *Po-hth* eRNAi *P. opilio* embryos (*Sharma et al., 2015*), again indicating that the asymmetric defects often obtained by the alternating temperature protocol are consistent with more localized gene expression perturbations.

Postembryos with schistomely or in 'Other abnormalities' category

Appendage development relies on differentiation along proximal-distal (P-D), dorsal-ventral (D-V), and anterior-posterior (A-P) axes, the last especially little studied in spiders. Genes involved

406 with establishing these axes may be susceptible to thermally-induced abnormal expression,
407 resulting in limb malformations. For example, a key player in establishing the D-V axis is the
408 gene *FoxB*, encoding a forkhead box transcription factor that is ventrally expressed within
409 appendages (*Heingård, 2017*; *Heingård et al., 2019*). Its knockdown in *P. tepidariorum* by
410 pRNAi resulted in greatly reduced hatching success and altered expression of downstream genes
411 that normally show ventral (*wingless* (*Pt-wg*/*Wnt1*), *Pt-H15-2*), dorsal (*optomotor-blind* (*Pt-*
412 *omb*)), and distal (*decapentaplegic* (*Pt-dpp*)) expression within appendages, resulting in
413 'dorsalized' legs and pedipalps (*Heingård, 2017*; *Heingård et al., 2019*). Such *Pt-FoxB* pRNAi
414 embryos that were able to hatch successfully and progress to the 1st stadium exhibited distally
415 crooked legs and pedipalps, comparable to some postembryos included in our 'Other
416 abnormalities' category (Table 1). This category also included postembryos with significantly
417 shortened appendages, a phenotype that has also been observed in mildly affected *Pt-Sp6-9*
418 pRNAi embryos and postembryos, and has included asymmetric defects (*Königsmann et al.,*
419 *2017*; *Setton & Sharma, 2018*).

420 Appendage bifurcation, i.e., schistomely (Fig. 2E and Fig. 2F), in postembryos might also be
421 considered in terms of erroneous expression of genes modeling the appendage axes, with
422 schistomely representing distal duplication of the P-D axis (*Cotoras, Castanheira & Sharma,*
423 *2021*). Though functional data (e.g., RNAi) are lacking in chelicerates (*Cotoras, Castanheira &*
424 *Sharma, 2021*), expression data in *P. tepidariorum* for *dpp* (*Akiyama-Oda & Oda, 2003*) and
425 *wg*/*Wnt1* (*Janssen et al., 2010*), among other evidence from spiders and other arthropods
426 (*Pechmann et al., 2010*), are consistent with *dpp* and *wg/Wnt1* expression early in spider
427 appendage development initiating a gene cascade that generates the P-D axis (*Prpic et al., 2003*).
428 In legs and pedipalps, three distinct domains of expression establish the P-D axis via expression

of *Dll* distally, *dachshund-1* (*dac-1*) medially, and *exd-1*/*hth-1* proximally (*Prpic & Damen, 2004*; *Pechmann et al., 2010*). Disturbances in the normal expression of *dpp*, *wg*/*Wnt1*, or their downstream targets caused by thermal shocks may result in a duplication of the P-D axis. In a report of cheliceral schistomely in the spider *Tetragnatha versicolor* Walckenaer, 1841, *Cotoras, Castanheira & Sharma* (*2021*) suggested the defect might be replicated by introducing ectopic Dpp and Wg/Wnt1. The schistomely shown in Fig. 2E, at the distal end of a leg, suggests perturbations that included direct or indirect abnormality in *Dll* expression while the more proximal schistomely presented in Fig. 2F, on a noticeably wider appendage than the normal legs, potentially represents abnormal expression of *dpp*, *wg*/*Wnt1*, and *dac-1* (among other possibilities), the latter's expression coincident with the trochanter and femur (Abzhanov & Kaufman, 2000; Prpic et al., 2003; Prpic & Damen, 2004).

Postembryos exhibiting pedicel polymely

Arguably the most interesting cases from the perspective of evolutionary/developmental biology involve two individuals with an appendage on the pedicel (first segment of the opisthosoma, O1; in spiders, coincident with somite VII) that are presented in Fig. 3A and 3B. Appendages do not usually form on the O1 segment in spiders and such defects are rare even among *E. atrica* subjected to alternating temperatures as embryos. Within this segment, the principal Hox genes expressed are the two paralogs of *Antennapedia* (*Antp*) (*Damen et al., 1998*; *Khadjeh et al., 2012*; *Schwager et al., 2017*). Knockdown of *Antp-1* in *P. tepidariorum* (*Pt-Antp-1*) by pRNAi demonstrates that it is responsible for repressing the development of legs on the O1 segment (*Khadjeh et al., 2012*). At its most severe, this down-regulation of *Pt-Antp-1* resulted in sufficient de-repression of leg development in O1 that 10 walking legs formed; the usual eight plus a pair on the pedicel that were like the former morphologically and in lateral placement

except a little shorter and thinner (*Khadjeh et al., 2012*; replicated by *Setton & Sharma, 2018*). Expression of the genes that establish the P-D axis in legs (*Pt-exd-1*, *Pt-hth-1*, *Pt-dac-1*, *Pt-Dll*) was nearly identical between the ectopic O1 legs and normal L1-L4 legs. Moreover, expression of the Hox genes *Deformed-A* (*Pt-Dfd-A*) and *Sex combs reduced-B* (*Pt-Scr-B*; paralogs as designated in *Schwager et al., 2017*) within the 10 legs indicated that the ectopic legs on O1 were not homeotic copies of any of the normal walking legs but they were instead true O1 segment de-repressed legs (*Khadjeh et al., 2012*).

It is of interest that *Khadjeh et al.* (*2012*) obtained not only severely affected postembryos with a pair of complete legs on the pedicel following knockdown of *Pt-Antp-1*, but in more moderately affected individuals they observed only short leg-like projections on the pedicel. Further, in a triple pRNAi experiment (to suppress *Pt-Antp-1* and two other Hox genes), they obtained two postembryos with an incomplete appendage on just one side of the pedicel. They attributed this asymmetric ("mosaic") phenotype to the lesser quantity of each dsRNA that could be injected when attempting to inhibit three genes simultaneously, resulting in less effective suppression of *Pt-Antp-1*. This range of outcomes is again reminiscent of the results obtained when alternating temperatures are applied to embryos of *E. atrica*, where appendages may form on the pedicel symmetrically or only on one side (Fig. 3), and these appendages may exhibit little or considerable development, from a short, unsegmented projection to a segmented, essentially complete leg (*Jacuński, 1971*; *Jacuński, 1984*; *Jacuński & Templin, 1991*; this study). This suggests that the alternating temperature protocol has the potential to result in reduced expression, to varying extent, of *Ea-Antp-1* in the O1 segment.

There is a long history of embryological observations on spiders that indicates an ancestry in which appendages were present on somite VII (e.g., *Korschelt & Heider, 1890*; *Jaworowski,*

475 *1896*; *Janeck, 1909*; *Yoshikura, 1954*; *Yoshikura, 1955*; *Wolff & Hilbrant, 2011*). Principally,
476 this is indicated by a small, short-lived protuberance or patch, sometimes explicitly interpreted as
477 an incipient limb bud, appearing on each O1 hemisegment when the opisthosomal limb buds
478 develop. These transient O1 limb buds apparently do not form in all spider taxa (*Dawydoff,*
479 *1949*), however, as they have not been noted in some detailed embryological studies
480 (*Montgomery, 1909*; *Holm, 1940*; *Rempel, 1957*; *Mittmann & Wolff, 2012*; *Pechmann, 2020*). It
481 is notable that putative limb buds on O1 have been observed in *Heptathela* (*Yoshikura, 1954*;
482 *Yoshikura, 1955*), a member of the basal Mesothelae, as well as in several members of the
483 derived araneomorph RTA clade, to which *E. atrica* belongs (*Wheeler et al., 2017*).

484 Considering that small, transitory protrusions (potential appendages) may appear on the pedicel
485 (O1) segment in embryonic spiders, and that by use of targeted gene suppression (pRNAi) it is
486 possible to obtain appendages on the pedicel with the structure of walking legs that nevertheless
487 have their own O1 identity (*Khadjeh et al., 2012*), it might be worth reconsidering whether
488 somite VII, the pedicel, is indeed the first segment of the opisthosoma, as it is usually described,
489 rather than the last segment of the prosoma. This thought is stimulated by another result obtained
490 by *Khadjeh et al.* (*2012*); that limb repression also occurs as a normal part of development in the
491 O2 segment (somite VIII), but when the genes that redundantly promote this repression (*Pt-Antp-*
492 *1*, *Ultrabithorax-1* (*Pt-Ubx-1*)) are suppressed by double pRNAi, the ectopic appendages that
493 form on O2 appear far more vestigial than the legs induced to form on O1. This may reflect less
494 effective overall de-repression in O2 because of the repression redundancy present in O2, not
495 shared by O1, but it could also conceivably reflect an early euchelicerate ancestry in which
496 appendages on somites VII and VIII differed substantially in morphology, with those on VII
497 more limb-like and those on VIII more plate-like, suggestive of a border between tagmata. Such

498 a difference in appendage morphology has been interpreted for the Devonian euchelicerate
499 *Weinbergina* and is also seen in extant Xiphosurida (horseshoe crabs) (*Dunlop & Lamsdell,*
500 *2017*).

501 Applying Lamsdell's (2013:4) definition of a tagma as "…a distinct and discrete morphological
502 region that comprises a series of equivalently modified appendages that constitute a unit of
503 specific form...or sometimes function...", the traditional view of the O1 segment as part of the
504 spider opisthosoma seems appropriate. Both the normally legless condition of the pedicel and the
505 maneuverability it imparts to the rest of the opisthosoma (*Dunlop & Lamsdell, 2017*) suggest a
506 form and function more in keeping with those of the opisthosoma. In addition, during spider
507 embryogenesis, the germ band initially divides into the prosomal segments and a posterior
508 'segment addition zone' (SAZ) from which the opisthosomal segments, including O1,
509 subsequently derive in anterior-to-posterior sequence (*Schwager et al., 2015*). These differing
510 paths to segmentation in the two tagmata also favor an opisthosomal identity for the O1 segment.

511 On the other hand, *Lamsdell* (*2013*) and *Dunlop & Lamsdell* (*2017*) acknowledge that
512 establishing borders between tagmata can be difficult because the ends of a tagma and their
513 associated appendages may differ substantially from the rest of the tagma. The border between
514 prosoma and opisthosoma, with somite VII's questionable affiliation, is given as a prime
515 problematic example (*Dunlop & Lamsdell, 2017*). They review evidence from fossil and extant
516 chelicerates that supports a chelicerate groundplan in which somite VII is prosomal, as suggested
517 by *Stürmer & Bergström* (*1981*). This possibility is further supported by the potential for
518 appendages with leg-like morphology to develop on the spider pedicel, whether induced by
519 application of pRNAi or alternating temperatures, and, along with transitory limb bud formation
520 on the O1 segment in some spiders, suggests loss of somite VII appendages present in basal

euchelicerate ancestors of arachnids (*Dunlop & Lamsdell, 2017*). Thus, an interpretation of atavism for appendages developing on the pedicel in teratological spiders (*Jacuński, 1971*; *Jacuński, 1984*; *Jacuński & Templin, 1991*) remains valid. Also noteworthy is the observation that, in some chelicerates, walking leg segments (all or just L4), as well as the opisthosomal segments, are derived from the SAZ and, in one known instance (a mite), O1 segmentation precedes that of L4 (reviewed in *Schwager et al., 2015*). Thus, it seems the mechanism of segmentation during embryonic development does not necessarily provide a reliable means for assigning segments to tagmata in a way that agrees with morphological/functional regions.

Summary and future directions

By applying alternating temperatures during early spider embryogenesis, we obtained high embryo mortality, changes in number, size, and shape of appendages or their podomeres, and formation of appendages on the pedicel; a body segment (O1 = somite VII) on which appendages are not normally found in spiders. Thus, by using appropriate methods, abnormalities can be induced that potentially reflect certain ancestral traits present in basal (eu)chelicerates, including possibly atavistic appendages on segment O1. This type of developmental abnormality has a bearing on the question of the tagma to which somite VII belongs, prosoma or opisthosoma, with implications tied to chelicerate phylogeny.

Based on recent research on genes that determine the formation of segments and appendages, we suspect that at least some of the observed developmental defects arising from our alternating temperature protocol are the result of blocked or otherwise aberrant expression of relevant genes, including Hox genes. Atypical expression may potentially include spatial and temporal, as well as quantitative, deviations from normal. Though the possible involvement of specific genes as

discussed above is speculative, it is one step toward the goal of testing hypotheses that attribute specific anomaly types to disturbances affecting specific genes. For example, by identifying *hb* as a candidate gene that may have its expression distorted by the alternating temperature protocol, potentially resulting in oligomely (as discussed above), the expression of *hb* over time may be compared between experimental and control embryos to ascertain if the former exhibit notable deviations in expression (e.g., asymmetric expression) compared to the latter. Modified versions of the alternating temperature protocol can also be investigated that intentionally attempt to disrupt expression of a specific gene and/or increase defect frequency; for example, by narrowing the window of treatment and exploring the application of an abrupt temperature switch at various times relative to the height of expression for a given gene and given site(s) within embryos. This could lead to the establishment of a protocol that is able to induce certain types of anomalies with greater regularity. Such an ability to more consistently generate certain defects would increase the feasibility of monitoring a gene's expression over time by reducing the number of embryos required.

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571 Author Contributions

572 Teresa Napiórkowska designed the research strategy, performed the experiments, analyzed the
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574 Julita Templin collected spiders for this research, prepared figures, and approved the final draft.

575 Paweł Napiórkowski collected spiders for this research, prepared the manuscript for revision, and
576 approved the final draft.

577 Mark Townley authored and reviewed drafts of the paper and approved the final draft.

578 Data Availability

579 The raw data is available in the table and figures.

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Table and Figure legends

Table 1. Types and frequency of anomalies in *Eratigena atrica* postembryos of the experimental group (i.e., subjected to the alternating temperature protocol). No defects were observed in postembryos belonging to the control group.

Figure 1. *Eratigena atrica* postembryo from control group, normally developed (ventral view). Ch, chelicera; L1-L4, walking legs 1-4; OP, opisthosoma; PL, pedicel; Pp, pedipalp; PR, prosoma.

Figure 2. *Eratigena atrica* postembryos with teratologic changes (ventral view). **A**: postembryo lacking right chelicera and with a protuberance (‘a’) on the gnathocoxa of the right pedipalp; **B**:

926 postembryo with abnormally developed right chelicera (‘a’) and lacking right pedipalp; **C**:
927 postembryo lacking one of the right walking legs; **D**: postembryo lacking right pedipalp and one
928 of the left walking legs; **E**: postembryo with schistomely of right second walking leg (L2), with
929 its free ends of similar length labeled ‘a’ and ‘b’; **F**: postembryo with deformed fourth walking
930 leg (L4) on left side of the prosoma, with its shortened free ends labeled ‘a’ and ‘b’. Ch,
931 chelicera; L1-L4, walking legs 1-4; Pp, pedipalp.

932 **Figure 3.** Ventral view of two *Eratigena atrica* postembryos with a short appendage on the
933 pedicel (left **A** and **B**). This appendage is enclosed by a white circle and shown enlarged (right **A**
934 and **B**). Small protuberances on the pedicel appendage in **A** are labeled ‘a’ and ‘b’ (right).
935 Contrary to the impression perhaps given by the image, these protuberances are not fused to L4.
936 Ch, chelicera; L1-L4, walking legs 1-4; Pp, pedipalp.

Table 1(on next page)

Types and frequency of anomalies in *Eratigena atrica* postembryos of the experimental group (i.e., subjected to the alternating temperature protocol). No defects were observed in postembryos belonging to the control group.

Table 1 Types and frequency of anomalies in *Eratigena atrica* postembryos of the experimental group (i.e., subjected to the alternating temperature protocol). No defects were observed in postembryos belonging to the control group.

Kind of anomaly	Number of individuals	%
Oligomely	117	55.19
Heterosymely	12	5.66
Schistomely	6	2.83
Bicephaly	7	3.30
Symely	3	1.41
Polymely	2	0.95
Complex anomalies	28	13.21
Other abnormalities	37	17.45
Total	212	100.00

Figure 1

Eratigena atrica postembryo from control group, normally developed (ventral view).

Ch, chelicera; L1-L4, walking legs 1-4; OP, opisthosoma; PL, pedicel; Pp, pedipalp; PR, prosoma.

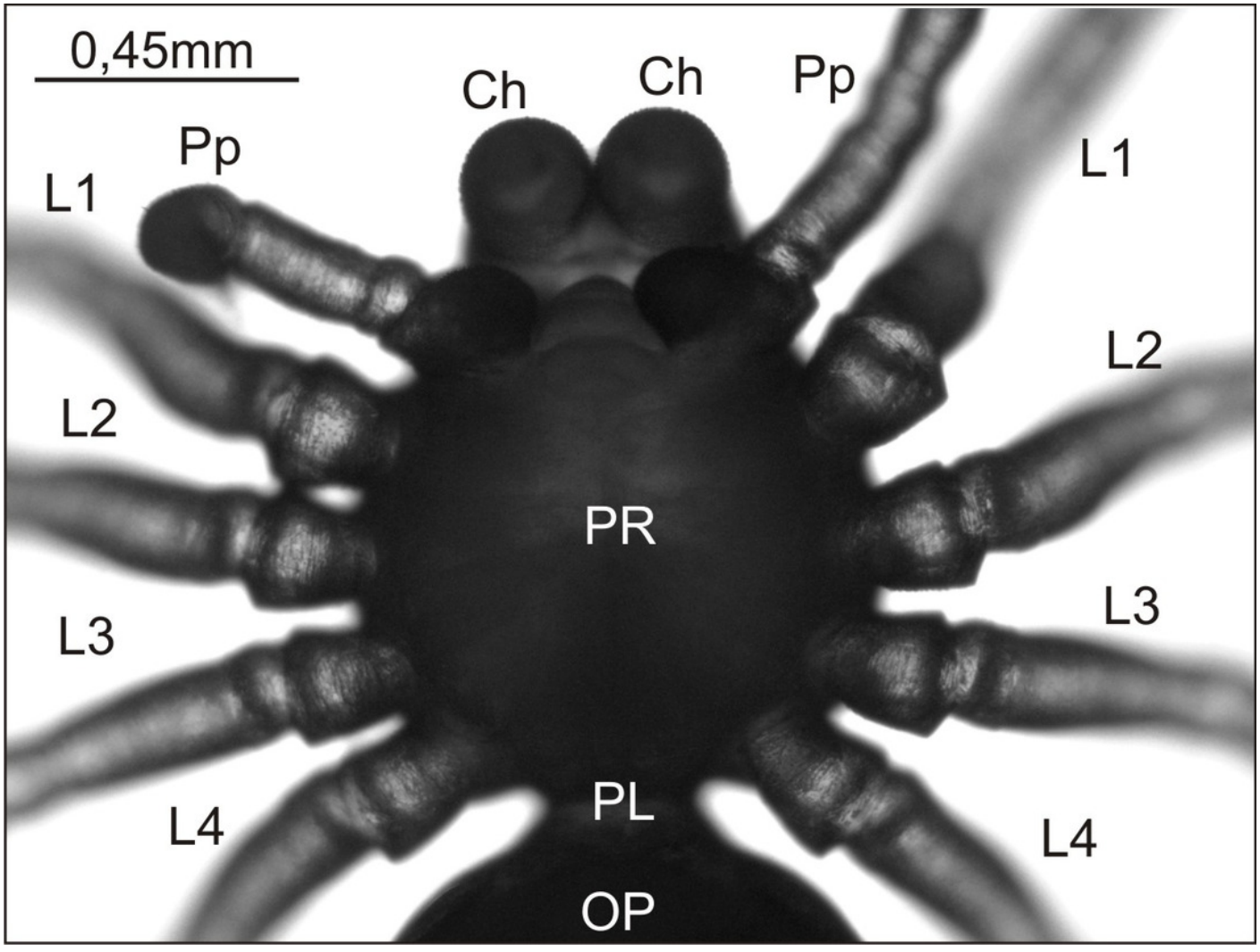

Figure 2

Eratigena atrica postembryos with teratologic changes (ventral view).

A: postembryo lacking right chelicera and with a protuberance (‘a’) on the gnathocoxa of the right pedipalp; **B**: postembryo with abnormally developed right chelicera (‘a’) and lacking right pedipalp; **C**: postembryo lacking one of the right walking legs; **D**: postembryo lacking right pedipalp and one of the left walking legs; **E**: postembryo with schistomely of right second walking leg (L2), with its free ends of similar length labeled ‘a’ and ‘b’; **F**: postembryo with deformed fourth walking leg (L4) on left side of the prosoma, with its shortened free ends labeled ‘a’ and ‘b’. Ch, chelicera; L1-L4, walking legs 1-4; Pp, pedipalp.

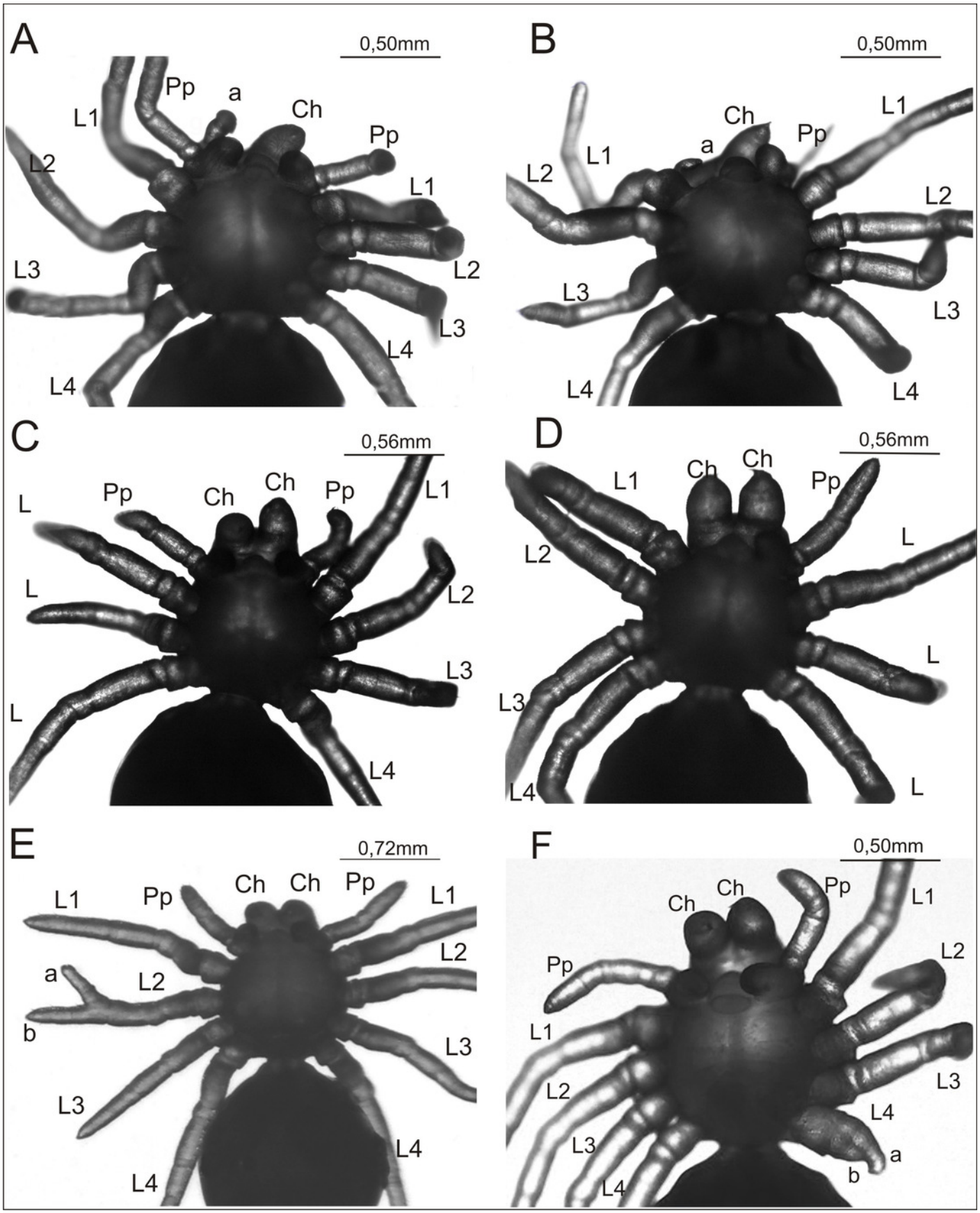
A
0,50mm
Pp
a
Ch
L1
Pp
L2
L1
L3
L2
L3
L4
L4
B
0,50mm
L1
Ch
Pp
a
L1
L2
L2
L3
L3
L4
L4
C
0,56mm
Pp
Ch
Ch
Pp
L1
L
L
L2
L3
L
L4
D
0,56mm
L1
Ch
Ch
Pp
L2
L
L
L3
L4
L
E
0,72mm
L1
Pp
Ch
Ch
Pp
L1
L2
a
L2
b
L3
L3
L4
L4
F
0,50mm
Ch
Ch
Pp
L1
Pp
L2
L1
L2
L3
L3
L4
a
L4
b

Figure 3

Ventral view of two *Eratigena atrica* postembryos with a short appendage on the pedicel (left A and B).

This appendage is enclosed by a white circle and shown enlarged (right **A** and **B**). Small protuberances on the pedicel appendage in **A** are labeled ‘a’ and ‘b’ (right). Contrary to the impression perhaps given by the image, these protuberances are not fused to L4. Ch, chelicera; L1-L4, walking legs 1-4; Pp, pedipalp.