There and back again: the dynamic evolution of panarthropod germ cell specification mechanisms

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Abstract

Germ cells enable the reproduction of an organism and the continuity of its lineage. These crucial cells are segregated from the soma at different times and places and via distinct mechanisms across the animal tree of life. Making sense of the evolution of germ cell specification across animals is complicated by the difficulty of making meaningful comparisons across the diversity of animal embryonic development. Here, we characterize germ cell specification within an ancient clade, that encompasses massive animal biodiversity but within which we can conduct meaningful comparative embryogenesis: the Panarthropoda. We amass data from centuries of studies describing the timing and mechanisms of germ cell formation, and apply ancestral state reconstruction to these data to propose novel hypotheses about the trajectory of evolution in this process. Furthermore, we speculate on the mechanisms underlying these evolutionary dynamics by considering the relationships among germ cell specification, concurrent developmental processes, and the germ line gene network. Collectively, this study derives new insights from a rich historical database of embryological observations, and has broad implications for understanding the evolution of metazoan germ cells.

Keywords: germ plasm, pole cells, inductive signaling, BMP, cytoplasmic determinant

I. Introduction

During development, animals specify the germ line, a collection of cells that will later give rise to the next generation. The specification and function of germ cells across animals has intrigued researchers for at least two millennia (Aristotle) . Whereas historical researchers relied primarily on observational data alone (Bounoure, 1939; Wolff, 1962) , we can now investigate these processes using functional genetic tools. These new techniques allow us not only to identify germ cells with more certainty, but also to gain more insight into the molecular mechanisms of germ cell specification. Several hypotheses have been proposed to explain evolutionary transitions between these mechanisms across animals (Buss, 1982; Buss, 1983; Crother et al., 2007; Crother et al., 2016; Evans et al., 2014; Extavour, 2007; Johnson et al., 2011; Whittle and Extavour, 2016; Whittle and Extavour, 2017) . However, such hypotheses are difficult to test because embryonic development of animals is too different across vast evolutionary time scales to be meaningfully compared. Moreover, there is disagreement in the field about whether existing well-studied mechanisms of specification are truly the only modes, and whether they are discrete, mutually exclusive mechanisms. Further, there are conflicting reports about the impact of germ cell specification mode on the evolution of genomes, body plans, and fitness (Evans et al., 2014; Otto and Hastings, 1998; Queller, 2000; Whittle and Extavour, 2016; Whittle and Extavour, 2017). Here, we examine variation in germ cell specification across the most speciose phylum of animals, wherein the general features of embryogenesis can nevertheless be meaningfully compared: the Panarthropoda. This clade displays striking diversity in the temporal and spatial origin of germ cells, as well as multiple transitions between

62 specification mechanisms, offering an ideal system to study evolution and regulation of
63 germ cell specification.
64 In this study, we first review the existing observational data regarding embryonic
65 germ line development across panarthropods. We are primarily interested in the first
66 embryonic appearance of germ cells before they enter the gonad, during which time we
67 refer to them as *primordial germ cells*. We identify three categories of temporally distinct
68 developmental context in which primordial germ cells are first detectable during
69 embryogenesis, and analyze the distribution of these three categories in their
70 phylogenetic context. We then review perturbational experimental studies aimed at
71 uncovering the mechanisms of germ cell specification. Finally, we discuss the
72 implications of these data on our understanding of the evolution of germ cell
73 specification in panarthropods, and more broadly across Metazoa.
74
75 ***I.i. How do we identify germ cells?***
76 Historically, embryologists studied early development using histological
77 preparations of sectioned embryos (His, 1868) . With light microscopy, primordial germ
78 cells can be readily distinguished from surrounding somatic cells by their rounder and
79 larger nuclei (Nieuwkoop and Sutasurya, 1979). The chromatin of germ cells often
80 appears more diffuse than that of somatic cells, and the cytoplasm, especially cortical or
81 perinuclear cytoplasm, may contain electron-dense, non-membrane bound granular
82 material (Eddy, 1975). However, tracking these cells throughout development using
83 fixed samples and morphological features alone is not always technically possible.

84 Since the late 20th century, studies assessing gene expression patterns unique to
85 or diagnostic of germ cells (henceforth referred to as “molecular markers”) have been
86 instrumental in identifying germ cells at earlier stages than previously recognized. The
87 most commonly used markers are the mRNA or protein products of the genes *vasa*,
88 *nanos*, *tudor*, and *piwi* (detectable by *in situ* hybridization or antibody staining,
89 respectively) (Ewen-Campen et al., 2010). The use of molecular markers can enable
90 germ cell identification at earlier developmental stages, since some germ cells express
91 their encoding genes before their morphological differentiation becomes unambiguously
92 detectable (see for example Ewen-Campen et al., 2013a) . In addition, these markers
93 facilitate tracking of germ cells over the course of development. However, using
94 molecular markers is a biased approach, as it relies on previous knowledge of germ line
95 gene expression from other species. Furthermore, interpretation of molecular marker
96 data can be complicated by pleiotropy, as their encoding genes may also be expressed
97 by somatic cells (e.g. Yajima and Wessel, 2011).
98
99 **II. Germ cell formation in panarthropods**
100 In our review of the embryological literature from 1860 to the present, we
101 identified three categories of primordial germ cell specification in panarthropods, based
102 on the timing of specification during embryogenesis (Figure 1). We describe these
103 categories below, along with relevant details of embryogenesis for context.
104

105 ***II.i. Category 1: PGC specification at early cleavage and blastoderm stages***

106 After fertilization, embryogenesis begins with *cleavage*, a series of mitotic
107 divisions of the zygote (Gilbert, 1997). In most arthropods, cleavage leads to the
108 formation of the *blastoderm*, a relatively uniform layer of cells at the egg cortex,
109 surrounding a central yolk mass populated by a small number of yolk cells (Anderson,
110 1973; Scholtz and Wolff, 2013). The first category of primordial germ cell specification in
111 panarthropods occurs during or before the formation of the blastoderm (Figure 1A).

112

113 *Insects*

114 In most insects, the blastoderm is initially *syncytial*, with no cell membranes
115 separating the nuclei from each other (Anderson, 1973; Johannsen and Butt, 1941). In
116 some insects, the primordial germ cells are the first cells to form, appearing at the
117 posterior pole before the rest of the blastoderm is cellularized (Figure 1A1). Primordial
118 germ cells that appear in this manner are called *pole cells*. Pole cells are most
119 commonly seen in species of Diptera (flies, midges and mosquitoes), Coleoptera
120 (beetles), and Hymenoptera (bees, ants and wasps), although they have also been
121 reported in Thysanoptera (thrips) and Dermaptera (earwigs) (Table S1).

122 Pole cells were first reported in species of the midge *Chironomus* by Weismann
123 (Weismann, 1863), although Metschnikow (1866) was the first to follow their migration
124 into the gonad. In many insects, the cytoplasm at the posterior pole of the embryo is
125 morphologically distinct from that of the rest of the embryo, often with visible granules
126 (Hegner, 1914; Zissler, 1992). The close association of this granular cytoplasm with
127 pole cells led researchers to speculate that the granular material contained germ line

128 determinants (e.g. Hegner, 1909a; Zissler, 1992); we will describe experimental data
129 testing this hypothesis in Part III below. It was long believed that pole cells gave rise to
130 somatic cell types in addition to the germ cells (Anderson, 1962a; Anderson, 1962b;
131 Poulson, 1947; Poulson, 1950). However, careful lineage tracing in the fruit fly
132 *Drosophila melanogaster* showed that all pole cells are determined as germ cells, and
133 any that become lost during their migration die (Underwood et al., 1980).
134 While the function of pole cells as primordial germ cells appears conserved, the
135 specific details of their formation can vary considerably across species. For example,
136 pole cells differ in which cleavage cycle they cellularize. Pole cells form during the ninth
137 mitotic cycle of *D. melanogaster* (Huettner, 1923) and during the sixth mitotic cycle in
138 the parasitic wasp *Nasonia vitripennis* (Buchta et al., 2013). To our knowledge, the
139 earliest observed formation of pole cells is during the third cleavage cycle in the midges
140 *Miastor metraloas* and *Chironomus sp*. (Hegner, 1914; Weismann, 1863). The number
141 of pole cells formed is also variable: a single pole cell forms in embryos of the latter
142 midges (Hegner, 1914; Weismann, 1863), whereas four appear in the fruit fly
143 *Bactrocera tryoni* (Anderson, 1962a) and sixteen appear in the beetle *Calligrapha*
144 *multipunctata* (Hegner, 1909b).
145 After the pole cells form, they enter the egg and migrate to the location of the
146 future embryonic gonad, which is quite far from the posterior pole (Santos and
147 Lehmann, 2004) . Several different migration pathways have been described. In *D.*
148 *melanogaster* and many other Diptera, the pole cells are carried into the egg during
149 posterior midgut invagination, then migrate through the epithelium of the posterior
150 midgut to enter the abdominal mesoderm and associate with the developing somatic

151 gonad (Metschnikow, 1866; Sonnenblick, 1941). In Hymenoptera, pole cells are
152 internalized with the rest of the mesoderm during gastrulation (Gatenby, 1917). Yet
153 another variation is observed in Chrysomelidae (leaf beetles) (Hegner, 1909a), where
154 germ cells are internalized with the rest of the germ band as the extraembryonic
155 membranes fold over the germ band.
156
157 *Other arthropods*
158 Primordial germ cells are also specified early in Collembola (springtails), an
159 early-diverging branch of non-insect hexapods (Du et al., 2024). Embryogenesis in
160 these animals begins with a period of holoblastic cleavage, and certain blastomeres on
161 the basal (yolk) side of the blastoderm can be recognized as likely primordial germ cells
162 very early in development (Jura, 1967; Klag, 1982). The cytoplasm of these cells
163 contains granular material visible by light and electron microscopy (Klag, 1982),
164 resembling the cytoplasm containing germ line determinants observed in insects
165 (Hegner, 1914; Zissler, 1992). Rather than maintaining contact with the germ band as in
166 insects, collembolan germ cells migrate into the center of the yolk after their
167 specification until eventually migrating to associate with the abdominal mesoderm, from
168 which the embryonic gonad develops (Jura, 1967).
169 Among non-hexapod Pancrustacea, embryos of Copepoda (copepods),
170 Amphipoda (beach hoppers), Decapoda (crabs, lobsters and shrimp), and some
171 Cladocera (water fleas) undergo stereotypical *holoblastic cleavage*, allowing precise
172 tracking of cell lineages and the identification of a specific cleavage blastomere as the
173 progenitor of primordial germ cells (Gerberding et al., 2002; Hertzler, 2005; Kühn, 1913;

174 McClendon, 1907). In amphipods and decapods, a putative germ plasm has been
175 identified in the freshly laid egg, termed the RNA-containing body (Gupta and Extavour,
176 2013) or intracellular body (Biffis et al., 2009; Chen et al., 2021). During cleavage, this
177 region of cytoplasm is inherited asymmetrically by the blastomeres that will give rise to
178 the primordial germ cells (Gupta and Extavour, 2013) (Figure 1A2).
179 In chelicerates (spiders, scorpions, ticks and mites), candidate primordial germ
180 cells are first detectable with molecular markers in the yolk of blastoderm-stage
181 embryos in the mite *Tetranychus urticae* (Dearden et al., 2003). As the germ band
182 forms, the primordial germ cells associate with the posterior germ band near the
183 primordia of the fourth walking legs (Dearden et al., 2003). We are not aware of any
184 other chelicerate or myriapod (centipedes and millipedes) species in which primordial
185 germ cell specification has been proposed to occur before gastrulation.
186
187 ***II.ii. Category 2: PGC specification at gastrulation***
188 After formation of the blastoderm, a portion of the blastoderm is specified as the
189 presumptive embryonic region, variably referred to as the *germ band*, *germ rudiment*,
190 *embryonic rudiment*, or *germ disc* depending on its initial form (Anderson, 1973;
191 Johannsen and Butt, 1941). The rest of the blastoderm differentiates into one or more
192 extraembryonic membranes (Johannsen and Butt, 1941). Soon after this specification,
193 *gastrulation* occurs, whereby some of the blastoderm cells are internalized and become
194 the mesoderm (Stern, 2004). In insects, mesoderm internalization primarily occurs
195 along the ventral midline of the germ band (Leptin, 2004; Roth, 2004). In other
196 arthropods, mesoderm may be internalized through a *blastopore* near the germ

197 rudiment (Chaw et al., 2007; Gerberding and Patel, 2004), or by epiboly (Edgar et al.,
198 2015). The second category of primordial germ cell differentiation occurs at or very
199 close to the time of gastrulation (Figure 1B).
200
201 *Insects*
202 Among insects, this timing of primordial germ cell specification is most common
203 among members of Hemiptera (true bugs), Psocodea (booklice), and Lepidoptera
204 (moths and butterflies), although it is also reported in species of Dermaptera, Blattodea
205 (cockroaches), Hymenoptera, Megaloptera (alderflies), Strepsiptera (twisted-wing flies),
206 Coleoptera, Mecoptera (scorpion flies), and Diptera. We observed three different spatial
207 patterns of germ cell specification around the time of gastrulation: before germ band
208 formation, at the posterior of the germ band after its formation, or at the ventral midline
209 of the blastoderm just before gastrulation. In Hemiptera, Psocodea, and Tenebrionidae
210 (darkling beetles; Coleoptera), germ cells are first detectable at the ventral posterior
211 region of the cellular blastoderm in an invaginating structure called the *posterior pit*
212 (Figure 1B1) (Butt, 1949; Goss, 1952; Mellanby, 1935a; Schröder, 2006; Shinji, 1919).
213 These cells appear to form on the side of the blastoderm epithelium facing the yolk,
214 rather than on the outside of the blastoderm like the pole cells described in Category 1.
215 In Hemiptera and Psocodea, the invagination continues into the egg towards the
216 anterior, forming a definitive germ band with germ cells at its posterior tip (Figure 1B1).
217 Germ cells in tenebrionid beetles move further inside the embryo once the amnion
218 (ventral extraembryonic membrane) has enclosed the entire ventral surface of the germ
219 band, similar to the internalization seen in coleopteran species that specify their germ

220 cells as pole cells (e.g. the weevils *Callosobruchus maculatus* (Brauer, 1925; Brauer,
221 1949; Quan, 2018) and *Acanthoscelides obtectus* (Jung, 1966; Lynch et al., 2011)).
222 Following their formation, the germ cells migrate along the dorsal side of the germ band
223 toward the anterior, eventually settling into the somatic gonad in the abdominal
224 mesoderm.

225 In several species with this timing of germ cell specification, primordial germ cells
226 were first identified at the posterior tip of the germ band after its detachment from the
227 blastoderm [*e.g.*, *Periplaneta orientalis* (Blattodea) (Heymons, 1895), *Sialis mitsuhashii*
228 (Megaloptera) (Suzuki et al., 1981), *Nematus ribesii* (Hymenoptera) (Singh, 1967), and
229 Mecoptera (Suzuki, 1990)]. Early studies of tenebrionid beetles described this pattern of
230 germ cell differentiation as well (Rempel and Church, 1969; Ullmann, 1964). However,
231 later work using molecular markers suggests that primordial germ cells in at least one
232 such beetle, *Tribolium castaneum*, appear earlier, during the cellular blastoderm stage
233 in a posterior pit much like the Hemiptera (Schröder, 2006). This raises the possibility
234 that in the other tenebrionid species listed above, primordial germ cells may likewise
235 differentiate from the blastoderm, with the reported later appearance in the germ band
236 simply reflecting limitations in detection methods.

237 In contrast to the posterior pit, germ cells differentiate along the ventral midline of
238 the blastoderm in Lepidoptera, separating from the blastoderm before gastrulation to lie
239 on the dorsal side of the presumptive germ band in the presumptive abdominal region
240 (Ando and Tanaka, 1980; Miya, 1958a; Presser and Rutschky, 1957; Tanaka, 1987;
241 Woodworth, 1889). Unlike when germ cells form at the posterior pit or at the posterior
242 tip of the germ band, in this case there does not appear to be long-range germ cell

243 migration, since the germ cells apparently differentiate in the same abdominal location
244 as the gonad.
245
246 *Other arthropods*
247 Among non-insect Pancrustacea, primordial germ cells are the first cells to be
248 internalized from the blastopore lip in Mysida (opossum shrimp) (Manton, 1928),
249 Anomopoda (water fleas) (Cannon, 1921), and the copepod orders Siphonostomatoida
250 (sea lice) (McClendon, 1907), Harpacticoida (Witschi, 1934), and Cyclopoida
251 (Schimkewitsch, 1896). In Isopoda (woodlice and pill bugs), primordial germ cells
252 differentiate from the mesendoderm soon after internalization (Figure 1B2) (Goodrich,
253 1939; Needham, 1942; Strömberg, 1965; Wolff, 2009). Thus, primordial germ cell
254 differentiation seems to be tied to gastrulation in these crustacean orders.
255 In chelicerates, primordial germ cells have been identified based on
256 morphological criteria at the blastopore in Opiliones (harvestmen) (Figure 1B3)
257 (Faussek, 1888; Faussek, 1892; Holm, 1947) and Scorpiones (Brauer, 1894), and a
258 recent study using molecular markers supported this interpretation (Gainett et al., 2022).
259 Putative primordial germ cells have also been identified at the blastopore in the
260 centipedes *Scolopendra cingulata* and *Strigamia maritima* (Myriapoda) (Green and
261 Akam, 2014; Heymons, 1901).
262 In Tardigrada (water bears), germ cells are the first cells to enter the blastopore
263 during gastrulation, based on data from both morphological characteristics (Hejnol and
264 Schnabel, 2005) and molecular markers (Heikes et al., 2023) (Figure 1B4). Lineage

265 tracing in these embryos suggests that the germ cells are derived from both
266 blastomeres of the two-cell embryo (Gabriel et al., 2007; Hejnol and Schnabel, 2005).
267 Finally, onychophoran (velvet worm) germ cells in different species emerge either
268 at the blastopore lip during gastrulation (Manton, 1949) or at the posterior end of the
269 germ band during elongation (Manton, 1949; Mayer and Tait, 2009; Sedgwick, 1887).
270 We are not aware of any studies using molecular markers to track germ cell formation in
271 Onychophora (Figure 1B5).
272
273 ***II.iii. Category 3: PGC specification from mesoderm***
274 After gastrulation, the germ band elongates and becomes segmented, and the
275 mesoderm becomes organized into pairs of somites in each segment. In most living
276 arthropods, each somite forms a small *coelom* enclosing a *coelomic cavity* (Anderson,
277 1973; Johannsen and Butt, 1941). The coelomic cavities are eventually lost as the
278 mesoderm differentiates into different cell types and reorganizes into the primordia of
279 the internal organ systems, including the somatic gonad (Anderson, 1973; Johannsen
280 and Butt, 1941). The final category of primordial germ cell differentiation timing
281 encompasses species in which primordial germ cells differentiate from the mesoderm at
282 this comparatively late stage of embryogenesis (Figure 1C).
283
284 *Insects*
285 In insects, this category includes members of Archaeognatha (jumping
286 bristletails), Zygentoma (silverfish and firebrats), Orthoptera (crickets and grasshoppers)
287 (Figure 1C1), Mantodea (praying mantises), Blattodea, Trichoptera (caddisflies),

288 Coleoptera, and Lepidoptera. Primordial germ cells may differentiate before formation of
289 the coelomic cavities [e.g., Orthoptera (Ewen-Campen et al., 2013b), Mantodea (Görg,
290 1959)], after formation of the coelomic cavities [e.g., Archaeognatha (Larink, 1969),
291 Zygentoma (Woodland, 1957)], or after the coelomic cavities are no longer recognizable
292 [e.g., Trichoptera (Miyakawa, 1974), *Odontotaenius disjunctus* (Coleoptera) (Krause,
293 1947), *Apis mellifera* (Hymenoptera) (Dearden, 2006; Nelson, 1915)]. In this
294 developmental timing category, primordial germ cells are usually specified in the same
295 abdominal segments where the somatic gonad forms, so there is no long-range
296 movement of primordial germ cells after their specification.
297
298 *Other arthropods*
299 Differentiation of primordial germ cells from the mesoderm has also been
300 described in Leptostraca (mud shrimp) (Figure 1C2) (Manton, 1934), Anaspidacea
301 (mountain shrimp) (Hickman, 1936), and Branchiopoda (fairy, clam and tadpole shrimp,
302 and water fleas) (Anderson, 1967; Mitsumoto and Makioka, 2002; Sudler, 1899).
303 However, data for stages of crustacean development late enough to include
304 differentiation of the trunk mesoderm from which primordial germ cells might arise are
305 sparse. This relative scarcity of early embryonic descriptions may be in part because
306 many crustaceans hatch as a nauplius, comprising only the head segments (Scholtz,
307 2000), and germ cells might not be specified until later larval stages, after formation of
308 the trunk. Many studies of naupliar and larval development in crustaceans are focused
309 on organogenesis of the gonad as a whole, without specific reference to the origin of

310 primordial germ cells (Cannon, 1924; Ikuta and Makioka, 1994; Mitsumoto and Makioka,
311 2002).
312 Classical studies on myriapods suggest a late differentiation of primordial germ
313 cells from the mesoderm in three of the four myriapod classes [Pauropoda (Tiegs,
314 1947), Symphyla (pseudocentipedes) (Tiegs, 1940), and Diplopoda (millipedes) (Dohle,
315 1964)], although only one species was reported from each class in these studies. Germ
316 cells in these classes have not been investigated with molecular markers to our
317 knowledge.
318 Among chelicerates, primordial germ cells differentiate from the mesoderm of the
319 opisthosomal segments in spiders (Figure 1C3) (Gough, 1902; Kautzsch, 1910;
320 Schimkewitsch, 1903). Data from molecular markers are consistent with this
321 observation (Schwager et al., 2015). Primordial germ cells are first detectable in the
322 mesoderm of the prosoma in pycnogonids (sea spiders) (Alexeeva and Tamberg, 2021;
323 Alexeeva et al., 2018; Miyazaki and Makioka, 2012).
324
325 ***II.iv. Summary: evolutionary trends from late to early germ cell specification***
326 In his monograph on embryogenesis in Dermaptera and Orthoptera, Richard
327 Heymons (Heymons, 1895) proposed that “the formation of sex cells at the rear end of
328 the embryonic primordium, which has now been clearly demonstrated for
329 representatives of very different groups, is probably generalizable for all insects.” Rather
330 than making broad statements such as this, we summarize the data regarding the
331 timing of germ cell specification (Table S1) in the context of currently accepted
332 panarthropod phylogeny (Ballesteros et al., 2022; Fernández et al., 2018; Giribet and

333 Edgecombe, 2019; Misof et al., 2014; Schwentner et al., 2017) in Figures 2 and 3. This
334 shows that in early diverging euarthropod orders, primordial germ cells tend to be first
335 observed later in embryogenesis (Category 3). The timing of first germ cell appearance
336 evolved to be earlier in development in multiple lineages independently (Dermaptera,
337 Condylognatha (true bugs and thrips), and Holometabola (undergoing complete
338 metamorphosis) within insects; Copepoda, Malacostraca (the largest class of
339 crustaceans), and Cladocera within non-hexapod Pancrustacea; Chilopoda (centipedes)
340 within myriapods; and Opiliones, scorpions, and mites within chelicerates). These data
341 are consistent with the hypothesis that differentiation from the mesoderm is the
342 ancestral mode of primordial germ cell specification in Euarthropoda.
343 Conflicting interpretations in the literature about the timing of germ cell
344 specification have sometimes resulted from technical advances enabling identification of
345 germ cells at stages earlier than previously detected. For example, early work on the
346 silk moth *Bombyx mori* (Lepidoptera) reported primordial germ cells emerging from the
347 mesoderm of coelomic cavities in late embryogenesis (Category 3) (Toyama, 1902),
348 while later studies identified primordial germ cells on the posterior side of the ventral
349 midline of the early germ band (Category 2) (Miya, 1953). The onset of primordial germ
350 cell specification before gastrulation was later further supported by data from molecular
351 markers (Nakao, 1999; Nakao et al., 2006). Therefore, it is possible that species that
352 are currently classified in Category 3 may be reclassified in Categories 2 or 1 by future
353 work deploying molecular markers. This caveat notwithstanding, we note that molecular
354 markers and functional genetic studies often corroborate late germ cell specification
355 [see for example *Gryllus bimaculatus* (Orthoptera, Insecta) (Ewen-Campen et al.,

356 2013b), *A. mellifera* (Hymenoptera, Insecta) (Dearden, 2006), *Parasteatoda*
357 *tepidariorum* (Araneae, Chelicerata) (Schwager et al., 2015)]. We therefore do not
358 assume that the hypothesis of ancestral late germ cell specification is an artifact of
359 exclusive use of morphological identification methods.

360 Going forward, data from key early diverging lineages should be gathered to
361 further test our hypothesis about the ancestral timing of primordial germ cell
362 specification. Given the phylogenetic position of the Palaeoptera (mayflies, dragonflies
363 and damselflies) as early diverging branches of the hexapod tree (Misof et al., 2014) ,
364 data about primordial germ cell specification in these orders would be especially useful
365 to test our hypothesis with respect to hexapods, as would more information from the
366 ametabolous Archaeognatha and Zygentoma or the non-insect hexapod Diplura (two-
367 pronged bristletails). Similarly, data about primordial germ cell specification are missing
368 from Oligostraca, the earliest diverging clade of Pancrustacea, which includes
369 Ostracoda (seed shrimp) and Branchiura (fish lice).

370 Outside of Pancrustacea, the chelicerate phylogeny remains unstable (Sharma
371 and Gavish-Regev, 2025), and embryological data are too scattered for us to suggest
372 trends in primordial germ cell specification within this subphylum. Data regarding
373 primordial germ cell differentiation are even sparser for myriapods: we are aware of
374 relevant data from only five species across the four myriapod classes (Dohle, 1964;
375 Green and Akam, 2014; Heymons, 1901; Tiegs, 1940; Tiegs, 1947). Studies of
376 primordial germ cells in a broader range of both chelicerates and myriapods, as well as
377 detailed embryological studies in unexplored taxa, will therefore be needed to fully

378 understand the developmental context and evolutionary trends of germ cell specification
379 in these subphyla.
380
381 **III. Molecular and genetic mechanisms of germ cell specification in**
382 **panarthropods**
383 Beyond studying where and when germ cells first appear during arthropod
384 embryogenesis, researchers have sought to understand how they are specified. Germ
385 cell specification in animals has historically been classified into two mechanisms:
386 maternal inheritance and zygotic induction (Extavour and Akam, 2003; Nieuwkoop and
387 Sutasurya, 1981). In maternal inheritance, germ cell determinants accumulate in a
388 specific region of the oocyte cytoplasm, forming a germ plasm. Cells that inherit
389 sufficient germ plasm components after the embryo divides become primordial germ
390 cells. In embryos that undergo zygotic induction, all cells are in principle competent to
391 become germ cells at the beginning of embryogenesis. Certain cells later receive
392 signals from neighboring cells that induce them to take on germ cell identity. In the
393 following section, we will describe how historical observational data and perturbational
394 experimental approaches have offered increasingly robust evidence to distinguish
395 between these mechanisms in several panarthropod species.
396
397 ***III.i. Maternal inheritance***
398 In the early twentieth century, researchers speculated that the electron-dense,
399 membraneless granules frequently found in the cytoplasm of primordial germ cells were
400 responsible for their specification. Experiments in chrysomelid beetles provided early

401 evidence that the posterior cytoplasm of the oocyte or early embryo contains germ cell
402 determinants. Burning or puncturing the posterior poles of beetle eggs with needles to
403 damage or remove posterior cytoplasm reduced or eliminated primordial germ cells
404 (Hegner, 1908; Hegner, 1911), demonstrating that this cytoplasm played a causal, and
405 not simply correlative, role in primordial germ cell specification. Studies in *D.*
406 *melanogaster* offered further evidence for the necessity of germ plasm. Removing or
407 destroying the posterior cytoplasm of *D. melanogaster* eggs via UV irradiation
408 demonstrated this region's requirement for germ cell formation (Geigy, 1931).
409 Subsequent centrifugation experiments in *D. melanogaster* embryos revealed that
410 shifting the granular structures in posterior cytoplasm away from the posterior pole
411 prevented germ cell formation or led to germ cells forming in a different part of the
412 embryo (Jazdowska-Zagrodzińska, 1966).
413 Experiments transplanting specific regions of cytoplasm between *D.*
414 *melanogaster* embryos showed that the contents of the posterior cytoplasm were
415 sufficient to specify germ cells. Transplanting posterior cytoplasm from undamaged *D.*
416 *melanogaster* embryos into sterile ones in which the posterior pole had been damaged
417 with UV irradiation rescued fertility, while transplanting anterior cytoplasm did not
418 (Okada et al., 1974). Further transplantation experiments demonstrated that posterior
419 pole plasm introduced into the anterior of another embryo caused formation of ectopic
420 pole cells that gave rise to functional germ cells when transplanted again to the
421 posterior of a third, sterile embryo (Illmensee and Mahowald, 1974). Similar
422 experiments transferring the posterior cytoplasm from late-stage oocytes into embryos
423 revealed that the oocyte pole plasm could specify functional primordial germ cells

424 (Illmensee et al., 1976). This result revealed that the necessary germ cell determinants
425 in *D. melanogaster* are deposited during oogenesis. These foundational studies
426 demonstrated that maternally derived cytoplasmic granules are both necessary and
427 sufficient for germ cell formation in some species.

428 Contemporary studies testing for maternal inheritance often ask whether known
429 germ line gene products accumulate during late oogenesis or early embryogenesis in
430 the region where primordial germ cells first arise. For example, antibody staining or *in*
431 *situ* hybridization experiments to detect germ line molecules like Vasa protein or *nanos*
432 mRNA have suggested the presence of germ plasm in mosquitoes (Juhn and James,
433 2006; Juhn et al., 2008), scuttle flies (Wotton et al., 2014), ants (Khila and Abouheif,
434 2008; Lynch et al., 2011; Rafiqi et al., 2020), wasps (Kharel et al., 2024; Lynch et al.,
435 2011), weevils (Lynch et al., 2011), aphids (Chang et al., 2006; Chang et al., 2007;
436 Chen et al., 2009; Lin et al., 2014), amphipods (Gupta and Extavour, 2013), and prawns
437 (Chen et al., 2021). Transcriptomic analyses of different portions of embryos and
438 oocytes (Carter et al., 2015; Quan and Lynch, 2016; Yoon et al., 2019) have also
439 revealed asymmetric localization of molecules associated with primordial germ cell
440 specification.

441 However, expression data showing asymmetric localization of putative germ
442 plasm components are not sufficient to demonstrate that these molecules function in
443 primordial germ cell specification. For this, functional genetic techniques are needed to
444 manipulate gene activity and test the effect on germ plasm assembly and germ line
445 development. The function of germ plasm components in panarthropods has been most
446 thoroughly studied in *D. melanogaster* (Lehmann, 2016; Mahowald, 2001; Trcek and

447 Lehmann, 2019). The membraneless ribonucleoprotein granules at the posterior of *D.*
448 *melanogaster* oocytes and early embryos are nucleated by the protein Oskar, which is
449 both necessary and sufficient for germ plasm assembly (Ephrussi and Lehmann, 1992;
450 Kistler et al., 2018). Oskar-mediated localization of core germ line molecules such as
451 Vasa, Tudor, Aubergine, and *nanos* to germ granules is essential for primordial germ
452 cell specification and maintenance of germ cell identity during cell migration and gonad
453 formation in these fruit flies (Lehmann, 2016). The roles of these germ plasm molecules
454 in establishing the *D. melanogaster* germ line have been extensively reviewed
455 elsewhere (Lehmann, 2016; Mahowald, 2001).

456 Analysis of germ plasm composition and localization in species beyond *D.*
457 *melanogaster* has revealed both conserved and rapidly evolving features. Within the
458 *Drosophila* genus, germ granule size, morphology (Counce, 1963; Mahowald, 1968;
459 Rivard et al., 2025), and the relative proportions of different granule components vary
460 widely (Doyle et al., 2023). The wasp *N. vitripennis* also has a well-characterized germ
461 plasm in the form of a single large structure called the oosome (Bull, 1982). Like *D.*
462 *melanogaster, N. vitripennis* requires *oskar* to assemble a germ plasm containing many
463 of the same molecules (e.g., Vasa, Tudor, and *nanos*) (Lynch and Desplan, 2010;
464 Lynch et al., 2011). However, while *D. melanogaster* has many small germ granules
465 (Mahowald, 1962), the *N. vitripennis* oosome has a distinct protein organization and
466 transcript composition compared to that observed in *D. melanogaster* (Kemph et al.,
467 2025; Kharel et al., 2024; Lynch et al., 2011). In several crustacean species, the germ
468 plasm appears as a large, round granule in the cortex of embryos and contains
469 conserved germ plasm markers (Chen et al., 2021; Grattan et al., 2013; Gupta and

470 Extavour, 2013). Studying germ plasm in additional species will be needed to clarify
471 whether and how variation in germ plasm makeup and organization influences the post-
472 transcriptional regulation and function of germ line determinants.
473
474 ***III.ii. Zygotic induction***
475 Experimentally demonstrating inductive germ cell specification is more
476 technically challenging than detecting germ plasm accumulation. Consequently, most
477 studies have inferred or hypothesized zygotic induction based on absence of evidence
478 of germ plasm. For example, *in situ* hybridization experiments fail to reveal asymmetric
479 localization of putative germ line gene transcripts in oocytes and early embryos of
480 various species, including milkweed bugs (Ewen-Campen et al., 2013a), crickets
481 (Ewen-Campen et al., 2013b), honeybees (Dearden, 2006), mites (Dearden et al.,
482 2003), spiders (Schwager et al., 2015), centipedes (Green and Akam, 2014), and
483 tardigrades (Heikes et al., 2023). Instead, key germ line genes are expressed in a
484 subset of cells later in embryogenesis, coincident with reports of the first primordial
485 germ cells based on morphological criteria (see Part II, Table S1). However, we note
486 that even in the well-accepted case of mouse primordial germ cell induction (Saitou and
487 Yamaji, 2010), it remains formally possible that some unknown, and therefore untested,
488 germ line determinant, or factor that makes cells competent to respond to later germ
489 line induction signals, is asymmetrically localized and preferentially inherited by germ
490 cells.
491 This caveat notwithstanding, there are multiple examples of experimental support
492 for induction rather than inheritance across panarthropods. Maternal knockdown of

493 putative germ plasm components in milkweed bugs and crickets reveals the
494 dispensability of maternal products of these genes for primordial germ cell formation
495 (Ewen-Campen et al., 2013a; Ewen-Campen et al., 2013b). Laser ablation of single
496 cells in two and four cell stage embryos of the tardigrade *Thuliana stephaniae* does not
497 prevent primordial germ cell specification, also suggesting that this species lacks a
498 cytoplasmic region with inherited germ line determinants (Hejnol and Schnabel, 2005).
499 Observations of germ cell formation in embryos with tissue duplications can be
500 interpreted as evidence against germ plasm and consistent with induction. For example,
501 in the drain fly *Clogmia albipunctata* and the flour beetle *T. castaneum*, genetic
502 manipulations producing double abdomen embryos lead to germ cell formation at both
503 anterior and posterior ends (Ansari et al., 2018; Yoon et al., 2019). Similarly, naturally
504 occurring “twins” of the stick insect *Carausius morosus* are duplicated embryos fused
505 along their anterior–posterior axis, that possess germ cells (Cavallin, 1971). In contrast,
506 double abdomen embryos of *D. melanogaster* only produce germ cells at the true
507 posterior, since germ plasm is absent at the ectopic posterior end (Lasko and
508 Ashburner, 1990).
509 Among panarthropods, experiments in the cricket *G. bimaculatus* have provided
510 the most direct evidence for zygotic induction of primordial germ cells. Knockdown of
511 the mesoderm specification factor *twist* leads to loss of primordial germ cells,
512 suggesting that mesoderm contributes to germ cell specification and/or maintenance
513 (Ewen-Campen et al., 2013b) and consistent with morphological evidence that
514 orthopteran primordial germ cells first arise from the embryonic mesoderm (Wheeler,
515 1893). The bone morphogenetic protein (BMP) signaling pathway, known to induce

516 germ cells in mice (Hopf et al., 2011; Lawson et al., 1999; Ying and Zhao, 2001; Ying et
517 al., 2000), is also required for cricket germ cell specification. Specifically, BMP signaling
518 and the downstream transcription factor Blimp-1 induce a subset of mesodermal cells to
519 become primordial germ cells in crickets (Donoughe et al., 2014; Nakamura and
520 Extavour, 2016).

521 The shared use of both BMP and Blimp-1 in mice and crickets may have
522 convergently evolved, or could suggest that this signaling mechanism is ancestral in
523 bilaterian inductive primordial germ cell specification (Lochab and Extavour, 2017).
524 Determining whether BMP and its downstream target genes are required for germ line
525 specification in more species will be necessary to distinguish between these
526 possibilities. Surprisingly few signaling pathways contribute to developmental fate
527 decisions in animals (Pires-daSilva and Sommer, 2003), increasing the likelihood of
528 evolutionary convergence, and limiting the number of candidates to evaluate for roles in
529 primordial germ cell induction in different species.

530

531 ***III.iii. Intermediate and flexible strategies of germ cell specification***

532 Evidence from some animal species suggests that there may not be a strict
533 dichotomy between inheritance and induction (discussed by Seervai and Wessel, 2013).
534 These mechanisms could instead represent two ends of a spectrum, and they may not
535 be mutually exclusive. Below we discuss cases of panarthropod species for which data
536 suggest that their mechanisms of germ cell specification may fall somewhere along this
537 continuum.

538 In the silk moth *B. mori*, historical studies have generated conflicting evidence
539 about how germ cells are specified. Germ granules were not detected in early light
540 microscopy experiments, but germ cells failed to form after cauterization of the ventral
541 side of cleavage stage embryos, suggesting this region could contain germ cell
542 determinants (Miya, 1958a). Subsequent studies found that Vasa protein is not
543 asymmetrically localized before cellularization, again suggesting a lack of germ plasm
544 (Nakao et al., 2006). However, the mRNA of one of four *B. mori nanos* homologs,
545 *nanosO*, localizes along the ventral midline of syncytial embryos immediately following
546 egg-laying, and is inherited by the primordial germ cells (Nakao et al., 2008).
547 Intriguingly, researchers found that maternal and zygotic *nanosO* act redundantly in
548 primordial germ cell specification; loss of one or the other reduces but does not
549 completely eliminate germ cells, whereas loss of both prevents germ cell formation
550 (Nakao and Takasu, 2019). The possibility that both inheritance and induction
551 mechanisms contribute to *B. mori* primordial germ cell specification suggests that this
552 species could offer insight into transitions in germ cell specification mechanisms within
553 Lepidoptera or in insects more broadly.
554 Although *D. melanogaster* is traditionally characterized as a species using a
555 germ plasm, zygotic signaling was recently suggested to contribute to germ cell
556 specification in this species (Colonnetta et al., 2022). Loss-of-function mutations in the
557 BMP family morphogen Dpp lead to partial loss of transcriptional quiescence and
558 mislocalization of germ plasm in the newly formed pole cells (Colonnetta et al., 2022).
559 Furthermore, loss of BMP signaling leads to activation of the normally suppressed
560 terminal signaling pathway in pole cells, which in turn disrupts their migration

561 (Colonnetta et al., 2022). These observations illustrate a universal conceptual challenge
562 in the field of developmental biology: distinguishing between, or even consistently
563 defining, cell type specification and maintenance. Although pole cells can form upon
564 disruption of BMP signaling, they do not contain the proper molecular components to
565 act as primordial germ cells (Colonnetta et al., 2022). We suggest it may be most useful
566 to define primordial germ cell specification mechanisms as those that ensure both the
567 physical formation of germ cell precursors (including adoption of specific morphologies
568 and activation of characteristic gene regulatory states), and the competence of those
569 cells to take the next developmental steps toward joining the gonad. Under this
570 definition, we would indeed have to concede that both germ plasm and zygotic inductive
571 mechanisms play roles in *D. melanogaster* germ cell specification.

572 The ability to post-embryonically regenerate a germ line initially specified via
573 maternal inheritance has also been reported in multiple invertebrate species, including
574 the sea squirt *Ciona intestinalis* (Takamura et al., 2002), the earthworm *Enchytraeus*
575 *japonensis* (Tadokoro et al., 2006), and the marine worm *Capitella teleta* (Dannenberg
576 and Seaver, 2018) (see also Özpolat, 2024 for a review of this phenomenon in
577 Annelida). The wasp *Pimpla turionellae* (Hymenoptera) has an oosome (Meng, 1968)
578 required for germ cell specification: damaging this material results in no pole cells,
579 which are the primordial germ cells of this species (Achtelig and Krause, 1971).
580 However, oosome-deficient embryos lacking pole cells develop into larvae with germ
581 cells in their gonad, indicating that an additional mechanism must regenerate germ cells
582 later in development (Achtelig and Krause, 1971). Evidence of germ line regeneration
583 has also been reported in the amphipod *Parhyale hawaiensis* (Kaczmarczyk, 2014) ,

584 which has a germ plasm that is asymmetrically segregated to the *g* micromere and its
585 lineage (Extavour, 2005; Gerberding et al., 2002; Gupta and Extavour, 2013). Ablation
586 of the *g* micromere eliminates primordial germ cells in the embryo (Extavour, 2005), but
587 the germ line is replaced post-embryonically with germ cells that appear to derive from
588 the mesoderm (Kaczmarczyk, 2014). The authors of this study speculated that these
589 replacement germ cells specifically derive from cells of the somatic gonad
590 (Kaczmarczyk, 2014), reminiscent of a theory popular for the first half of the 20th
591 century, that primordial germ cells in most animals derived from the inner epithelium of
592 the somatic gonad (Everett, 1944; Hargitt, 1919; Heys, 1931). Species capable of
593 regenerating primordial germ cells present the opportunity to investigate the relative
594 contributions of and potential interactions between inheritance- and induction-based
595 mechanisms in a single species. Further, these species may represent living examples
596 of the intermediates that are hypothesized to exist in evolutionary transitions between
597 modes of germ line specification (Extavour, 2007).
598

599 **IV. Evolutionary drivers of panarthropod germ cell specification**

600 In the following section, we discuss the evolutionary dynamics of germ cell specification
601 mechanisms across Panarthropoda, and propose potential developmental and genetic
602 factors that might contribute to these dynamics.
603

604 ***IV.i. Convergent evolution of panarthropod germ plasm***

605 The phylogenetic distribution of germ cell specification mechanisms across
606 panarthropods reveals a complex evolutionary history. Data for tardigrades,

607 chelicerates, and myriapods are consistent with induction as the ancestral mechanism
608 of panarthropod primordial germ cell specification (Figure 3). Pancrustacea are the most
609 heavily represented group in our dataset (Table S1). The late origin of primordial germ
610 cells in several species suggests induction may be widely used, but there appear to be
611 independent origins of germ plasm in Malacostraca and Branchiopoda. The use of
612 induction and germ plasm in different decapod species suggests that further sampling
613 could reveal patterns in the evolution of germ cell formation within this order (Biffis et al.,
614 2009; Chen et al., 2021; Terao, 1929). However, functional experiments and broader
615 taxonomic sampling will be necessary to confirm mechanisms and reconstruct their
616 evolutionary trajectories across Panarthropoda as a whole.
617 Reminiscent of a broader pattern we and others previously reported across
618 animals (Extavour, 2007; Extavour and Akam, 2003; Johnson et al., 2003; Whittle and
619 Extavour, 2017), in some insect orders we noted that early diverging lineages deployed
620 induction, while later branching lineages appeared to use germ plasm (Figure 2, 3). This
621 pattern is well exemplified in Coleoptera, (Kobayashi et al., 2006; Komatsu and
622 Kobayashi, 2012; Krause, 1947; Niikura et al., 2017; Schröder, 2006; Ullmann, 1964),
623 with pole cells forming only in Chrysomeloidea (including long-horned and leaf beetles)
624 and Curculionidae (Brauer, 1925; Hegner, 1908; Lynch et al., 2011). These
625 observations raise the possibility that germ plasm may not be ancestral to Coleoptera.
626 When considered alongside the absence of germ plasm in most Lepidoptera, the
627 observation of late-arising, Category 3 germ cells in Trichoptera (Miyakawa, 1974) and
628 Category 2 germ cells in Mecoptera (Suzuki, 1990), and the distinct morphologies of *N.*
629 *vitripennis* and *D. melanogaster* germ plasm (see Part III.i), these patterns are

630 consistent with a novel hypothesis in which germ plasm evolved independently multiple
631 times across insects, rather than only once in the Holometabola as we and others have
632 previously proposed (Ewen-Campen et al., 2012; Ewen-Campen et al., 2013b; Lynch et
633 al., 2011). Our previous work had suggested the independent evolution of germ plasm
634 at the phylum level (Extavour and Akam, 2003), but this new hypothesis is the first, to
635 our knowledge, to suggest such widespread convergent evolution of maternal
636 inheritance-based germ cell specification within a phylum.

637 To test this hypothesis, we performed ancestral character reconstruction using
638 both maximum parsimony and maximum likelihood approaches. For this analysis, we
639 designated species as using inheritance or induction based on experimental evidence
640 when available or inferred from observational data describing the timing and location of
641 germ cell specification (Supplementary Materials, Table S1). The results of a parsimony
642 analysis inferred that the ancestral panarthropod specification mode was induction, with
643 a minimum of 24 changes over the history of the panarthropods (Figure S1; Table S3).
644 When considering all equally parsimonious character histories, changes from induction
645 to maternal inheritance (min = 18, max = 23, average = 20.5) were the most common,
646 but reverse transitions were also inferred (min = 2, max = 7, average = 4.5). At a
647 minimum, reversions from maternal inheritance to induction were inferred in the
648 honeybee *A. mellifera* (Hymenoptera) and the drain fly *C. albipunctata* (Diptera). We
649 note that all maximum parsimony reconstructions assign induction as the ancestral state
650 of Holometabola (Figure S1, node 11), which requires many fewer total transitions
651 within Holometabola (N = 12) than would be required if germ plasm evolved a single

652 time in the last common ancestor of Holometabola (N = 20) as previously proposed
653 (Ewen-Campen et al., 2013b; Lynch et al., 2011) .
654 Marginal ancestral reconstructions of a maximum likelihood approach also
655 proved consistent with the parsimony analysis described above (see Table S3 for a
656 comparison), again providing strong statistical support for induction as the ancestral
657 specification mechanism in Panarthropoda, Arthropoda, and Insecta, with multiple
658 independent instances of germ plasm evolution and reversion to induction (Figure S2;
659 see Table S7 for marginal probabilities). We further find strong evidence that induction
660 was the ancestral specification mechanism in Holometabola (probability 80.6%),
661 followed by independent transitions to germ plasm in the common ancestors of Diptera
662 and Mecoptera (or independently within Mecoptera, Siphonaptera (fleas), and Diptera),
663 derived lineages of Lepidoptera, in the common ancestor of Chrysomelidae and
664 Curculionidae (Coleoptera), and in the common ancestor of Hymenoptera. Outside of
665 Holometabola, we infer independent transitions to germ plasm in Thysanoptera,
666 Hemiptera, Dermaptera, Collembola (which may have undergone multiple independent
667 transitions within the class), Decapoda, the last common ancestor of Hyalidae and
668 Talitridae (Amphipoda), and in the chelicerate *Tetranychus urticae*. Collectively, these
669 qualitative and quantitative analyses suggest a previously unappreciated dynamism in
670 the evolutionary history of germ cell specification within Panarthropoda.
671
672 ***IV.ii. The relationship between germ cells and somatic germ layers***
673 In arthropods with late formation of primordial germ cells (Category 3), primordial
674 germ cells appear to differentiate directly from the mesoderm (Evans, 1901; Hickman,

675 1936; Nelson, 1915). Consistent with this hypothesis, abolishing mesoderm prevents
676 germ cell specification in the cricket *G. bimaculatus* (Ewen-Campen et al., 2013b).
677 Germ cell differentiation occurs concurrently with the differentiation of many other
678 mesodermal cell types, including the somatic gonad, musculature, heart, and fat body
679 (Johannsen and Butt, 1941). We suggest that the types of mechanisms that specify
680 germ cells are not distinct in principle from those specifying any other mesodermal cell
681 type in these animals.

682 The germ layer origin of germ cells is less clear in arthropods whose germ cells
683 appear before or very close to the time of gastrulation (Category 2). For example,
684 primordial germ cells in many crustaceans are described as being the first to enter the
685 blastopore during gastrulation (e.g. Cannon, 1921; Goodrich, 1939; Manton, 1928), and
686 are usually considered by researchers to be derived from the mesendoderm. In
687 contrast, germ cells in Category 2 insects arise before gastrulation (Mellanby, 1935b;
688 Ullmann, 1964), consistent with the hypothesis that germ cells are distinct from the
689 mesoderm and may not derive from any germ layer.

690 In crustaceans in which a putative germ plasm has been identified, cleavage
691 patterns are stereotypical, and cell lineages are available, the germ cell lineage is sister
692 to the mesoderm or the mesendoderm [Amphipoda (Extavour, 2005; Gerberding et al.,
693 2002; Scholtz and Wolff, 2002; Wolff and Scholtz, 2002), Euphausiacea (krill) (Alwes
694 and Scholtz, 2014), Decapoda (Hertzler, 2005)], but never to the ectoderm. In addition,
695 removing the putative germ plasm from embryos of the amphipod *P. hawaiensis* makes
696 the *g* micromere morphologically indistinguishable from the other micromeres, which
697 give rise to mesoderm or endoderm in unperturbed embryos (Gupta and Extavour,

698 2013). This result suggests that in the absence of germ cell determinants, the *g*
699 micromere, normally the germ line progenitor, could instead adopt mesendodermal fate.
700 This hypothesis has not been directly tested in this amphipod because gastrulation fails
701 after ablation of the putative germ plasm (Gupta and Extavour, 2013). Together, these
702 data suggest a close association between the germ cell and mesendodermal lineages in
703 these crustaceans. This relationship is apparent beyond Pancrustacea, since germ cells
704 induced late in embryogenesis arise from the mesoderm (Category 3). We thus
705 speculate that germ cells in panarthropods may be ancestrally derived from the
706 mesodermal lineage, consistent with our inference that inductive germ cell specification
707 is ancestral to panarthropods (see Part IV.i above).
708

709 ***IV.iii. Evolutionary shifts in the spatial and temporal origin of primordial germ***
710 ***cells***

711 We have seen significant variation in the spatial and temporal origin of primordial
712 germ cells across panarthropods (Figures 2–3; Table S1). However, regardless of when
713 primordial germ cells first appear, we find a common stage across many panarthropods
714 in which primordial germ cells are situated in bilateral segmental clusters across
715 multiple trunk segments. Soon after this stage, the segmental clusters on each side fuse
716 together into bilaterally symmetrical genital ridges, corresponding to the primordial
717 embryonic gonad (Miya, 1958b; Nelsen, 1934; Seidel, 1924; Ullmann, 1964).
718 Most arthropods undergo “short germ” development, wherein the blastoderm
719 tissue generates only anterior segments, with posterior segments being added
720 sequentially to the posterior end of the germ band later in development (Krause, 1939) .

721 In contrast, the “long germ” mode of development seen in *D. melanogaster*, wherein the
722 blastoderm tissue immediately generates all body segments, is an evolutionary novelty
723 found only in some insects (Clark et al., 2019; Krause, 1939). In Category 1 or 2
724 arthropods with short germ development, germ cells are often specified before the
725 formation of the segments that will include the future embryonic gonad. In Category 2
726 insects, germ cells often associate with the posterior end of the germ band and then
727 migrate toward the anterior as the germ band elongates (Goss, 1953; Hegner, 1909b;
728 Heming and Huebner, 1994; Paz, 1958; Suzuki, 1990; Ullmann, 1964). This migratory
729 path is so common among insects that Johanssen and Butt included it in their
730 description of embryogenesis in a stereotypical insect (Johannsen and Butt, 1941).
731 The association of germ cells with the posterior end of the embryo may have had
732 other evolutionary consequences in insects. Many insects, both long and short germ,
733 specify the body plan early in development via maternal deposition of axial patterning
734 determinants, independent of the mechanism of germ cell specification (reviewed in
735 Lynch, 2019; Rosenberg et al., 2009). For example, the short germ grasshopper
736 *Schistocerca americana* exhibits asymmetric localization of the posterior patterning
737 determinant *nanos* in the absence of a germ plasm (Lall et al., 2003). However, insects
738 with germ plasm that includes axial patterning determinants, like *D. melanogaster*, often
739 exhibit long germ development. Based on these observations, we employed
740 phylogenetic logistic regression and Pagel’s test of evolutionary rate covariation (Pagel,
741 1994) to test the hypothesis that the acquisition of long germ band development
742 influences germ cell mechanism transitions or vice versa, and found statistically
743 significant evidence of this association using both methods (Figure S3; Figure S4). All

744 Pagel covariation models assuming coupled evolutionary rates among these two
745 characters are significantly better fits to the data than the null model of independent
746 evolution (likelihood ratio test p-value < 1e−3; Figure S4; Table S8). We note that the
747 bidirectional and GB-dependent models perform similarly to the GC-dependent model,
748 suggesting both that long germ band development may drive the evolution of germ
749 plasm, and that germ plasm may drive transitions to long germ band development.
750 We further hypothesize that a spatial shift in germ cell specification to the
751 posterior end of the embryo in Category 2 insects created a condition in which germ line
752 determinants are localized to the same embryonic region as posterior patterning
753 determinants (Figure 4B). Subsequently, germ line determinants may have hitchhiked
754 onto the asymmetric localization machinery, coupling germ cell specification to posterior
755 patterning and thus promoting the evolution of germ plasm (Figure 4C). Under this
756 hypothesis, the germ plasm of *D. melanogaster* would represent the final stage of this
757 evolutionary scenario, as its germ granules include both germ line and abdominal
758 patterning determinants (Ephrussi and Lehmann, 1992; Trcek et al., 2015; Wang and
759 Lehmann, 1991).
760 *N. vitripennis* is an intriguing case to study the relationship between
761 anteroposterior patterning and germ cell specification. The posterior cytoplasm of this
762 wasp has two populations of *nanos* mRNA: one that is incorporated into the oosome
763 and eventually into pole cells, and another that functions in axial patterning (Lynch and
764 Desplan, 2010). *nanos* localization to the oocyte posterior in this wasp depends on
765 actin-dependent anchoring (Olesnicky and Desplan, 2007), just as in *D. melanogaster*
766 (Forrest and Gavis, 2003), suggesting a conserved localization mechanism between

767 these two species. We speculate that *N. vitripennis* represents an evolutionary
768 intermediate between species where anteroposterior patterning is entirely independent
769 of germ cell formation (Figure 4A) and those where these two processes are fully linked
770 (Figure 4C). Our evolutionary hypothesis could be tested further by examining the
771 mechanisms of localization of posterior patterning determinants and germ line
772 determinants in more insect species to understand how existing cytoskeletal machinery
773 for one developmental process may have been coopted for use in another.
774

775 ***IV.iv. Potential genetic basis of evolutionary shifts in the molecular mechanism of***
776 ***primordial germ cell specification***

777 Beyond the timing and position of germ cell origin, evolutionary changes in the
778 localization of gene products and timing of gene expression may also underlie
779 transitions in the mechanism of specification (Extavour, 2007; Srouji and Extavour,
780 2010). Many of the genes important for specifying germ cells are conserved across
781 metazoans (Ewen-Campen et al., 2010), but they exhibit distinct temporal and spatial
782 expression patterns across species. In species that use inheritance, the germ line gene
783 products are expressed during oogenesis and persist in the early embryonic cytoplasm
784 to be inherited by the primordial germ cells. In species that induce germ cells, these
785 genes are either not expressed in oocytes or the gene products are neither
786 asymmetrically nor preferentially inherited by particular cells. As we have suggested
787 previously (Extavour, 2007; Srouji and Extavour, 2010), relatively simple regulatory
788 modifications affecting post-transcriptional regulation, the cytoskeleton, or RNA or

789 protein localization could cause germ line gene product retention in oocytes, thereby
790 enabling the transition from induction to inheritance.
791 The insect-specific gene *oskar* offers a potential example of gene network
792 evolution driving shifts in germ cell specification mechanism. Since *oskar* is likely a
793 nucleator of germ plasm in multiple insects (Ephrussi et al., 1991; Jones and
794 Macdonald, 2007; Kemph and Lynch, 2022; Lynch et al., 2011; Rivard et al., 2025) and
795 has been lost multiple times since its origin prior to the divergence of Pterogyta from
796 Ametabola (Blondel et al., 2021), we hypothesized that *oskar* gene presence in the
797 genome might be correlated with germ line specification mode in a given taxon (Figure
798 S3). A related hypothesis, that the evolutionary advent of the *oskar* gene facilitated the
799 evolution of holometabolous germ plasm, was previously proposed based on the co-
800 occurrence of *oskar* transcript and germ plasm in *N. vitripennis* and *D. melanogaster*, as
801 well as the absence of both *oskar* homologs and germ plasm in *T. castaneum*, *A.*
802 *mellifera*, *C. albipunctata*, most Lepidoptera, and the hemimetabolous genomic
803 resources available at the time (Lynch et al., 2011). The results of applying phylogenetic
804 logistic regression to this hypothesis (Figure S3; Table S9) suggest a strong and
805 statistically significant association between *oskar* presence and maternal inheritance
806 (coefficient = 2.5827, adjusted odds-ratio: = 13.28, p-value = 0.00059) We emphasize
807 that this observed association does not imply that *oskar* is necessary or sufficient for
808 germ plasm formation; indeed, there are insect species without *oskar* that nevertheless
809 have germ plasm and species with *oskar* that lack germ plasm (Figure S3), as we
810 discuss in Part IV.v below.

811 In contrast to prior hypotheses from our group and others of a single origin of
812 germ plasm at the base of the Holometabola (Ewen-Campen et al., 2012; Ewen-
813 Campen et al., 2013b; Kemph and Lynch, 2022; Lynch et al., 2011), our current
814 likelihood and parsimony reconstruction of induction as ancestral to Holometabola
815 (Figure 5; Figures S1–S2) implies that *oskar* has been independently recruited as a
816 germ plasm nucleator at least twice, in Hymenoptera (Lynch et al., 2011) and in Diptera
817 (Ephrussi and Lehmann, 1992). The strong association between *oskar* presence and
818 germ plasm (Figure S3) implies that it may have been co-opted for germ plasm
819 nucleation even more times across insects. We note that such “gene reuse” is a
820 common feature of convergent evolution across multiple biological processes (Chan et
821 al., 2010; Martin and Orgogozo, 2013; Morris, 2009; Reed et al., 2011; Satterlee et al.,
822 2024). Since *oskar* is co-expressed with germ plasm components in nervous systems of
823 hemimetabolous and holometabolous insects (Ewen-Campen et al., 2012; Kulkarni et
824 al., 2023; Xu et al., 2013), we posit that independent acquisitions of germ plasm in
825 distinct lineages may have proceeded via parallel cooption of a pre-existing gene
826 interaction network. Future studies should aim to determine whether each independent
827 origin of germ plasm in the Holometabola arose by co-option of *oskar* to the germ line,
828 or by *oskar*-independent mechanisms of germ plasm assembly.
829 The results of our analysis (Figure S3; Table S9) suggest that *oskar*’s functional
830 evolution may have contributed to a transition from induction to inheritance, but
831 precisely when and how *oskar* acquired a germ line role remains unclear. Morphological
832 data suggest that thrips use germ plasm (Haga, 1985; Heming, 1979), and *oskar*
833 homologs have been found in this order (Blondel et al., 2021). If *oskar* plays a role in

834 thrips germ cell specification, it could support the entirely novel hypothesis that *oskar*
835 evolved its germ line determinant properties before the evolution of complete insect
836 metamorphosis.
837
838 ***IV.v. The case for novel panarthropod germ plasm nucleators***
839 Recruitment of *oskar* to the germ line is not the only mechanism explaining the
840 evolution of germ plasm in arthropods. In the genomes of species of Carabidae (ground
841 beetles), an early diverging beetle family that lacks pole cells, *oskar* orthologs have
842 been identified, decoupling the presence of *oskar* from germ plasm in Holometabola
843 (Blondel et al., 2021; Kobayashi et al., 2013). Similarly, the genome of the
844 hemimetabolous pea aphid *Acyrthosiphon pisum* lacks an *oskar* ortholog but their
845 embryos have a germ plasm containing Vasa and Nanos proteins (Chang et al., 2006;
846 Chen et al., 2009; Lin et al., 2014), which are asymmetrically localized by unknown
847 mechanisms. Vasa is also asymmetrically localized to a putative germ plasm in
848 amphipod and decapod species (Chen et al., 2021; Gupta and Extavour, 2013), which
849 is also likely to have evolved via distinct mechanisms since *oskar* is an insect-specific
850 gene (Blondel et al., 2021). Outside of arthropods, germ plasm nucleators have
851 convergently evolved in diverse animal phyla (Aoki et al., 2016; Bontems et al., 2009;
852 Kulkarni and Extavour, 2017; Lee et al., 2020; Marlow and Mullins, 2008; Scholl et al.,
853 2024). Similarly, we posit that germ plasm nucleators other than *oskar* must also exist
854 across the panarthropods, awaiting discovery.

855

856 **V. Concluding thoughts**

857 By reinterpreting the meticulous work of generations of classical embryologists
858 through the lens of modern developmental genetics and an improved panarthropod
859 phylogeny, we have generated novel, testable evolutionary hypotheses about the
860 mechanisms that specify the germ line, with implications for the genetic basis of
861 convergence in evolution that extend beyond this superphylum. The advent of new
862 genetic tools in an increasing number of panarthropod model species places us in an
863 exciting era to tackle age-old questions about the evolution of panarthropod germ cell
864 specification, as well as the impact of these mechanisms of genome evolution and body
865 patterning in the most successful group of animals on earth.

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Data Availability

All data sources are listed in the accompanying Supplementary Material and/or are available at https://github.com/rishabhrajkapoor/panarthropoda_gc_specification_evolution, commit ID ff9945c.

Author Contributions

All authors gathered data and performed data analysis and interpretation. JAK, ELR and RRK wrote the first draft of the manuscript. CGE conceived of the study, obtained funding for the study, supervised its execution, and reviewed and edited the manuscript.

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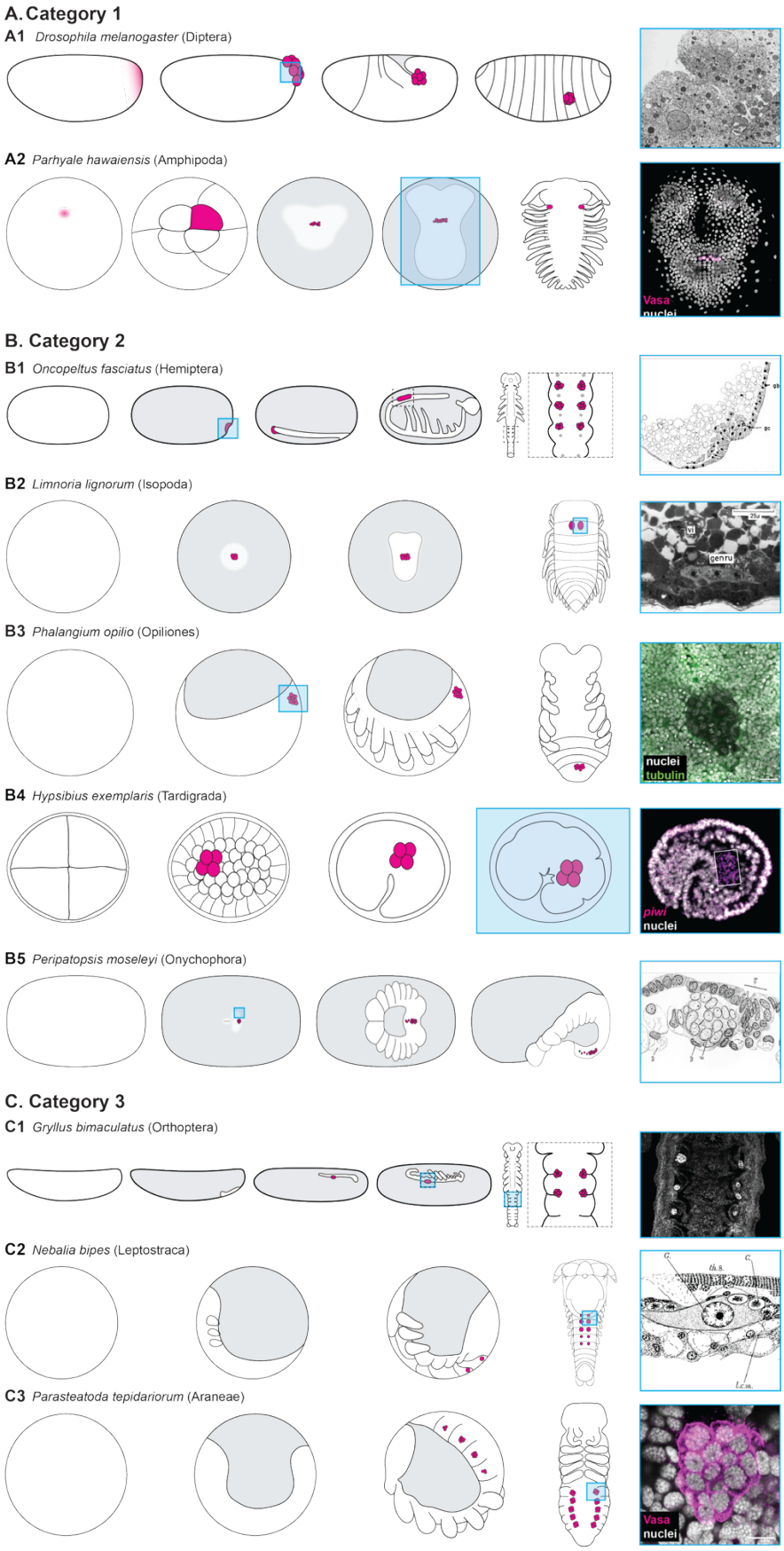
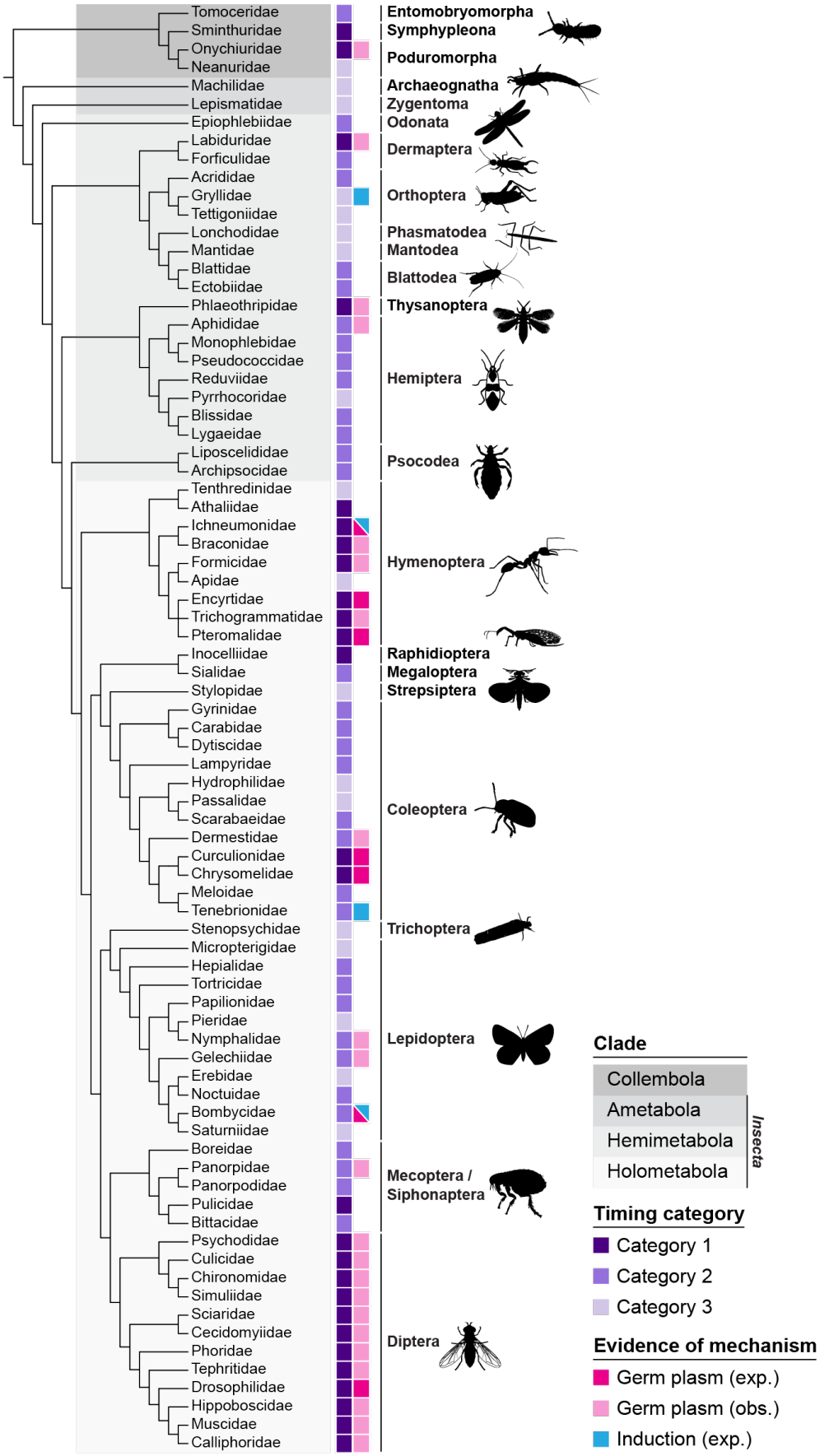
Conflicts of Interest

The authors declare no conflicts of interests.

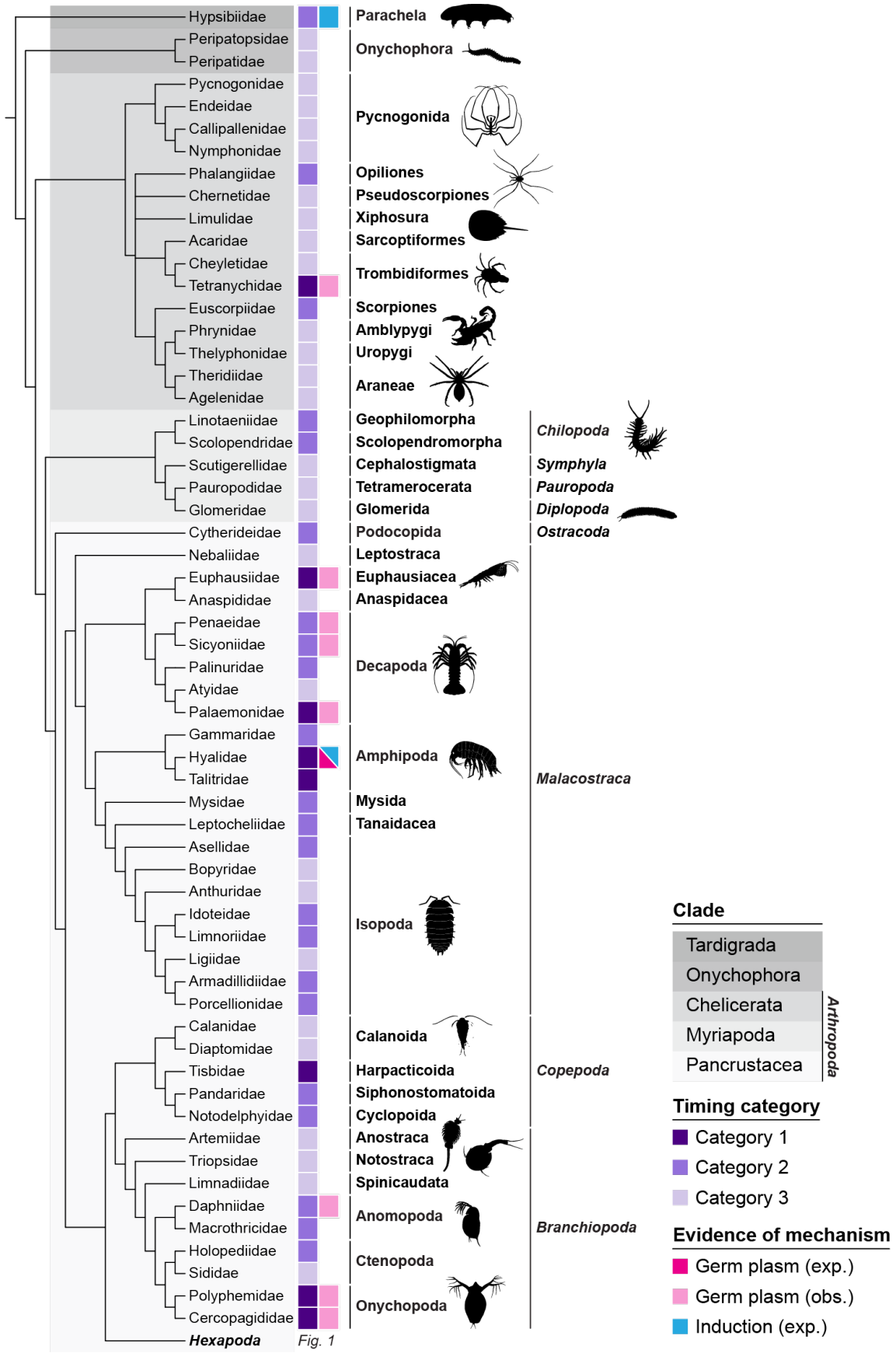

A. Category 1
A1 Drosophila melanogaster (Diptera)
A2 Parhyale hawaiensis (Amphipoda)
Vasa
nuclei
B. Category 2
B1 Oncopeltus fasciatus (Hemiptera)
B2 Limnoria lignorum (Isopoda)
vl
genru
B3 Phalangium opilio (Opiliones)
nuclei
tubulin
B4 Hypsibius exemplaris (Tardigrada)
piwi
nuclei
B5 Peripatopsis moseleyi (Onychophora)
C. Category 3
C1 Gryllus bimaculatus (Orthoptera)
C2 Nebalia bipes (Leptostraca)
C3 Parasteatoda tepidariorum (Araneae)
Vasa
nuclei

894 **Figure 1 (previous page). Primordial germ cell origin in the context of panarthropod**
895 **embryogenesis.** In all schematics, magenta represents the approximate location of germ plasm or
896 primordial germ cells; gray represents yolk or extraembryonic tissue, blue box in schematic shows the
897 stage/location where germ cells are detectable in the micrographs and drawings on the right. (**A1**)
898 Schematic: In the fruit fly *Drosophila melanogaster*, germ plasm accumulates at the posterior of syncytial
899 embryos and is inherited by pole cells that migrate to the ultimate site of the embryonic gonad (Illmensee
900 and Mahowald, 1974). Data: TEM micrograph displaying pole cells budding in *D. melanogaster* embryos.
901 The pole cell nuclei (N) have characteristically diffuse chromatin, and polar granules (P) are distributed
902 throughout the cytoplasm. Image reproduced with permission from (Mahowald, 1968). M: mitochondria;
903 N: nuclei; P: polar granules. (**A2**) Schematic: In the amphipod *Parhyale hawaiensis*, germ plasm
904 accumulates in a region of the embryo called the RNA-containing body, which is inherited by the *g*
905 micromere at the 8-cell stage (Extavour, 2005; Gupta and Extavour, 2013). All descendants of *g* are
906 primordial germ cells. Data: Primordial germ cells visualized by immunohistochemistry with a cross-
907 reactive antibody raised against recombinant Vasa protein from both *D. melanogaster* and the
908 grasshopper *Schistocerca americana* (Chang et al., 2002). (**B1**) Schematic: In the milkweed bug
909 *Oncopeltus fasciatus*, primordial germ cells first appear in the ventral posterior blastoderm in an
910 invaginating structure called the posterior pit (Ewen-Campen et al., 2013a). Primordial germ cells remain
911 at the posterior tip of the germ band as it invaginates into the yolk. The germ cells migrate along the germ
912 band toward the anterior to segments A4–A6. Data: Hand drawing of a light micrograph of a sectioned
913 late blastoderm stage *O. fasciatus* embryo showing germ cells in the posterior pit . Image reproduced with
914 permission from (Butt, 1949). gb: germ band; gc: germ cell. (**B2**) Schematic: In the isopod *Limnoria*
915 *lignorum*, primordial germ cells are first identifiable among the mesendodermal mass just after the
916 beginning of gastrulation (Strömberg, 1968). Primordial germ cells remain associated with the mesoderm
917 throughout development. Data: Light micrograph of a sectioned *L. lignorum* embryo showing primordial
918 germ cells distinguished by their lighter-staining cytoplasm and larger nuclei among the mesoderm at the
919 extended germ band stage. Image reproduced with permission from (Strömberg, 1968). vi: vitellophage;
920 gen ru: genital rudiment. (**B3**) Schematic: In the harvestman *Phalangium opilio*, primordial germ cells are
921 first identifiable at the posterior end of the germ band soon after the germ band forms (Faussek, 1888;
922 Gainett et al., 2022). Primordial germ cells remain at the posterior end of the germ band at least until the
923 fourth opisthosomal segment is formed, after which the germ cells can be found in that segment. Data: ;
924 Chromatin (visualized with the nucleic acid dye Hoechst 33342) is more diffuse in primordial germ cells
925 than in surrounding cells. Image kindly provided by Prashant Sharma (University of Wisconsin, Madison).
926 Green: alpha-tubulin. (**B4**) Schematic: In the tardigrade *Hypsibius exemplaris*, primordial germ cells are
927 first identifiable in the 32-cell stage and are the first cells to be internalized during gastrulation. Data:
928 Primordial germ cells visualized by *in situ* hybridization against *He-vasa*. Image kindly provided by Kira
929 Heikes (Duke University). (**B5**) Schematic: In the velvet worm *Peripatopsis moseleyi*, primordial germ
930 cells are first identifiable near the blastopore at the beginning of gastrulation. As the embryo elongates
931 and more segments form, the primordial germ cells migrate to more anterior segments. Data: Hand
932 drawing of a light micrograph of a *P. moseleyi* embryo showing primordial germ cells soon after their
933 internalization during gastrulation, distinguished by their lighter-staining nuclei and cytoplasm. Image
934 reproduced with permission from (Manton, 1949). g: germ cells; en: endoderm; b.a.: blastoporal area.
935 (**C1**) Schematic: In the cricket *Gryllus bimaculatus*, primordial germ cells are first detectable among the
936 mesoderm in segments A2–A4 at embryonic stage 6, after most abdominal segments have been
937 specified (Ewen-Campen et al., 2013b). Data: Primordial germ cells visualized by immunohistochemistry
938 with an antibody against *Gb*-Piwi. (**C2**) Schematic: In the leptostracan *Nebalia bipes*, primordial germ
939 cells are first identifiable in the mesoderm in the thorax and abdomen during the extended germ band
940 stage (Manton, 1934). Data: hand drawing of a light micrograph of a sectioned *N. bipes* embryo showing
941 primordial germ cells among the mesoderm in the extended germ band, distinguished by their lighter-
942 staining cytoplasm and larger nuclei. Image reproduced with permission from (Manton, 1934). G: genital
943 rudiment; l.c.m.: circular muscle round liver lobe; th.8: eighth thoracic segment. (**C3**) Schematic: In the
944 house spider *Parasteatoda tepidariorum*, primordial germ cells are first identifiable in segmental clusters
945 in the opisthosomal mesoderm after all segments have formed (Schwager et al., 2015). Data: Primordial
946 germ cells visualized by immunohistochemistry using an antibody raised against *Pt*-Vasa.


Tomoceridae
Sminthuridae
Onychiuridae
Neanuridae
Machilidae
Lepismatidae
Epiophlebiidae
Labiduridae
Forficulidae
Acrididae
Gryllidae
Tettigoniidae
Lonchodidae
Mantidae
Blattidae
Ectobiidae
Phlaeothripidae
Aphididae
Monophlebidae
Pseudococcidae
Reduviidae
Pyrrhocoridae
Blissidae
Lygaeidae
Liposcelididae
Archipsocidae
Tenthredinidae
Athaliidae
Ichneumonidae
Braconidae
Formicidae
Apidae
Encyrtidae
Trichogrammatidae
Pteromalidae
Inocelliidae
Sialidae
Stylopidae
Gyrinidae
Carabidae
Dytiscidae
Lampyridae
Hydrophilidae
Passalidae
Scarabaeidae
Dermestidae
Curculionidae
Chrysomelidae
Meloidae
Tenebrionidae
Stenopsychidae
Micropterigidae
Hepialidae
Tortricidae
Papilionidae
Pieridae
Nymphalidae
Gelechiidae
Erebidae
Noctuidae
Bombycidae
Saturniidae
Boreidae
Panorpidae
Panorpodidae
Pulicidae
Bittacidae
Psychodidae
Culicidae
Chironomidae
Simuliidae
Sciaridae
Cecidomyiidae
Phoridae
Tephritidae
Drosophilidae
Hippoboscidae
Muscidae
Calliphoridae
Entomobryomorpha
Symphypleona
Poduromorpha
Archaeognatha
Zygentoma
Odonata
Dermaptera
Orthoptera
Phasmatodea
Mantodea
Blattodea
Thysanoptera
Hemiptera
Psocodea
Hymenoptera
Raphidioptera
Megaloptera
Strepsiptera
Coleoptera
Trichoptera
Lepidoptera
Mecoptera / Siphonaptera
Diptera
Clade
Collembola
Ametabola
Hemimetabola
Holometabola
Insecta
Timing category
Category 1
Category 2
Category 3
Evidence of mechanism
Germ plasm (exp.)
Germ plasm (obs.)
Induction (exp.)

947

Figure 2 (previous page). Germ cell specification across Hexapoda. Phylogeny of studied hexapod lineages indicating the primordial germ cell origin timing category and the inferred mechanism of primordial germ cell specification. Phylogenetic relationships in the cladogram are derived from published relationships listed in Table S2; branch lengths are not to scale. Data on the timing of germ cell specification are shown in the first column as follows: dark purple = Category 1 (cleavage/blastoderm), medium purple = Category 2 (gastrulation), light purple = Category 3 (mesoderm differentiation). Data on the mechanism of germ cell specification, inferred as described in Supplementary Materials, are shown in the second column: dark pink = experimental evidence supports the use of germ plasm, light pink = morphological evidence of germ plasm, blue = experimental evidence of induction. Data and data sources are described in more detail in Table S1.


Hypsibiidae
Peripatopsidae
Peripatidae
Pycnogonidae
Endeidae
Callipallenidae
Nymphonidae
Phalangiidae
Chernetidae
Limulidae
Acaridae
Cheyletidae
Tetranychidae
Euscorpiidae
Phrynidae
Thelyphonidae
Theridiidae
Agelenidae
Linotaeniidae
Scolopendridae
Scutigerellidae
Pauropodidae
Glomeridae
Cytherideidae
Nebaliidae
Euphausiidae
Anaspididae
Penaeidae
Sicyoniidae
Palinuridae
Atyidae
Palaemonidae
Gammaridae
Hyalidae
Talitridae
Mysidae
Leptocheliidae
Asellidae
Bopyridae
Anthuridae
Idoteidae
Limnoriidae
Ligiidae
Armadillidiidae
Porcellionidae
Calanidae
Diaptomidae
Tisbidae
Pandaridae
Notodelphyidae
Artemiidae
Triopsidae
Limnadiidae
Daphniidae
Macrothricidae
Holopediidae
Sididae
Polyphemidae
Cercopagididae
Hexapoda
Parachela
Onychophora
Pycnogonida
Opiliones
Pseudoscorpiones
Xiphosura
Sarcoptiformes
Trombidiformes
Scorpiones
Amblypygi
Uropygi
Araneae
Geophilomorpha
Scolopendromorpha
Cephalostigmata
Tetramerocerata
Glomerida
Podocopida
Leptostraca
Euphausiacea
Anaspidacea
Decapoda
Amphipoda
Mysida
Tanaidacea
Isopoda
Calanoida
Harpacticoida
Siphonostomatoida
Cyclopoida
Anostraca
Notostraca
Spinicaudata
Anomopoda
Ctenopoda
Onychopoda
Chilopoda
Symphyla
Pauropoda
Diplopoda
Ostracoda
Malacostraca
Copepoda
Branchiopoda
Arthropoda
Clade
Tardigrada
Onychophora
Chelicerata
Myriapoda
Pancrustacea
Timing category
Category 1
Category 2
Category 3
Evidence of mechanism
Germ plasm (exp.)
Germ plasm (obs.)
Induction (exp.)
Fig. 1

958

959 **Figure 3 (previous page). Panarthropod germ cell specification outside of Hexapoda.** Phylogeny of
960 studied non-hexapod panarthropod lineages indicating the primordial germ cell origin timing category and
961 the inferred mechanism of primordial germ cell specification. Phylogenetic relationships in the cladogram
962 are derived from published relationships listed in Table S2; branch lengths are not to scale. Data on the
963 timing of germ cell specification are shown in the first column as follows: dark purple = Category 1
964 (cleavage/blastoderm), medium purple = Category 2 (gastrulation), light purple = Category 3 (mesoderm
965 differentiation). Data on the mechanism of germ cell specification, inferred as described in Supplementary
966 Materials, are shown in the second column: dark pink = experimental evidence supports the use of germ
967 plasm, light pink = morphological evidence of germ plasm, blue = experimental evidence of induction.
968 Data and data sources are described in more detail in Table S1.

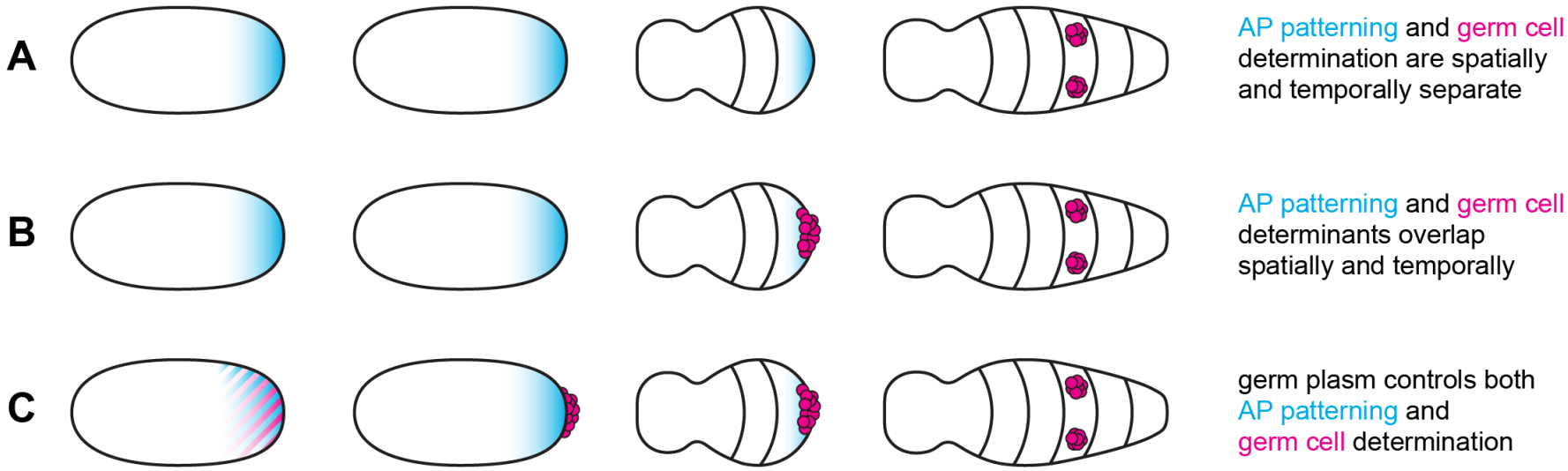


Figure 4. Hypothesized links between spatial and temporal dynamics of germ cell origination and the mechanism of germ cell specification. (**A**) In species that induce germ cells later in embryogenesis (Category 3), the asymmetrically localized anterior–posterior (AP) patterning determinants (blue) and germ cell determination factors (pink) are spatially and temporally separate. (**B**) Proposed shift to earlier germ cell specification at the embryonic posterior in Category 2 insects enabled overlap between the patterning and germ cell determinants. (**C**) Subsequent hitchhiking of the asymmetric localization machinery by germ line determinants could have coupled germ cell specification and posterior patterning and thus promoted the evolution of germ plasm.

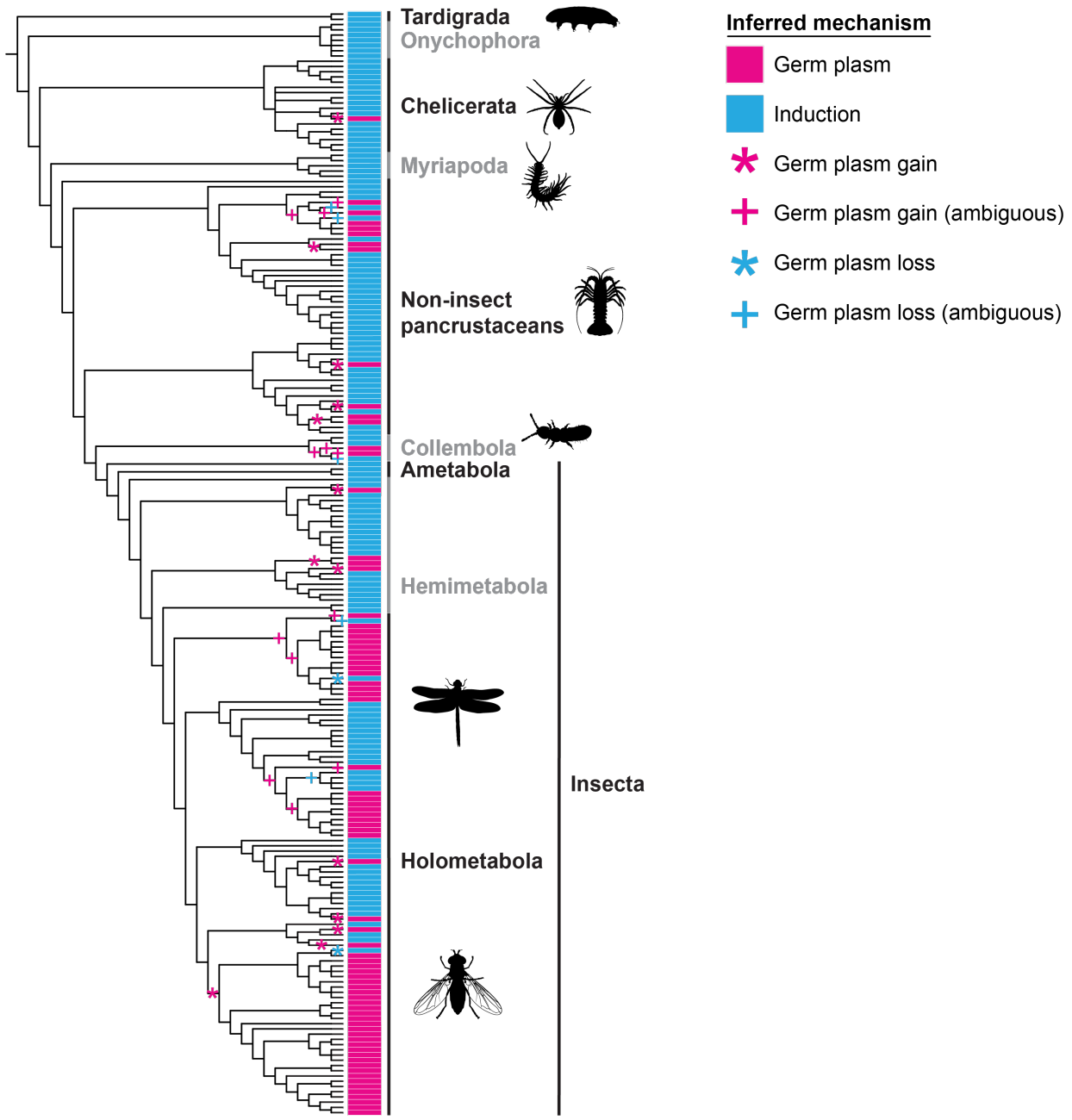


Figure 5. Evolutionary dynamics of panarthropod germ cell specification. Phylogenetic relationships among all study species (Table S1) are derived from published relationships listed in Table S2; branch lengths are not to scale. Colored lines to the right of each branch in the cladogram designate the mechanism of germ cell specification (inferred as described in Supplementary Materials) as follows: pink = germ plasm, blue = induction. Pink asterisks denote proposed instances of germ plasm evolution and pink pluses mark ambiguous cases of germ plasm gain. Blue asterisks denote proposed instances of germ plasm loss and blue pluses mark ambiguous cases of germ plasm loss.

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