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 a clade as old as all animals, which encompasses massive animal biodiversity but within which it is still possible to conduct comparative embryogenesis: the Panarthropoda. We amass data from centuries of studies describing the timing and mechanisms of germ cell formation, and use 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 process of germ cell specification across animals has intrigued researchers for more than 150 years. Whereas historical researchers relied primarily on observational data alone, 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. 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; Whittle and Extavour, 2016; Whittle and Extavour, 2017) . Here, we examine variation in germ cell specification across the most speciose phylum of animals, within which 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 specification mechanisms, offering an ideal system to study evolution and regulation of germ cell specification.

60 In this study, we first review the existing observational data regarding embryonic
61 germ line development across panarthropods. We are primarily interested in the first
62 embryonic appearance of germ cells before they enter the gonad, during which time
63 they are referred to as *primordial germ cells*. We identify three categories of temporally
64 distinct developmental context in which primordial germ cells are first detectable during
65 embryogenesis and analyze the distribution of these three categories in their
66 phylogenetic context. We then review perturbational experimental studies aimed at
67 uncovering the mechanisms of germ cell specification. Finally, we discuss the
68 implications of these data on our understanding of the evolution of germ cell
69 specification in panarthropods, and more broadly across Metazoa.
70
71 ***I.i. How do we identify germ cells?***
72 Historically, embryologists studied early development using histological
73 preparations of sectioned embryos. With light microscopy, primordial germ cells can be
74 readily distinguished from surrounding somatic cells by their rounder and larger nuclei
75 (Nieuwkoop and Sutasurya, 1979). The chromatin of germ cells often appears more
76 diffuse than that of somatic cells, and the cytoplasm, especially cortical or perinuclear
77 cytoplasm, may contain electron-dense, non-membrane bound granular material (Eddy,
78 1975). However, tracking these cells throughout development using fixed samples and
79 morphological features alone is not always technically possible.
80 Since the late 20th century, studies assessing gene expression patterns unique to
81 or diagnostic of germ cells (henceforth referred to as “molecular markers”) have been
82 instrumental in identifying germ cells at earlier stages than previously believed. The

83 most commonly used markers are the mRNA or protein products of the genes *vasa*,
84 *nanos*, *tudor*, and *piwi* (detectable by *in situ* hybridization or antibody staining,
85 respectively) (Ewen-Campen et al., 2010). The use of these molecular germ cell
86 markers can enable their earlier identification, since some germ cells express their
87 encoding genes before their morphological differentiation becomes detectable. In
88 addition, these markers facilitate tracking of germ cells over the course of development.
89 However, using molecular markers is a biased approach, as it relies on previous
90 knowledge of germ line gene expression from other species. Furthermore, interpretation
91 of molecular marker data can be complicated by the fact that their encoding genes are
92 sometimes also expressed by somatic cells (e.g. Yajima and Wessel, 2011).
93

94 **II. Germ cell formation in panarthropods**

95 In our review of the embryological literature from 1860 to the present, we
96 identified three categories of primordial germ cell specification in panarthropods, based
97 on the timing of specification during embryogenesis (Figure 1). We describe these
98 categories below, along with relevant details of embryogenesis for context.
99

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

101 After fertilization, embryogenesis begins with *cleavage*, a series of mitotic
102 divisions of the zygote (Gilbert, 1997). In most arthropods, cleavage leads to the
103 formation of the *blastoderm*, a relatively uniform layer of cells at the egg cortex,
104 surrounding a central yolk mass populated by a small number of yolk cells (Anderson,

1973; Scholtz and Wolff, 2013). The first category of primordial germ cell specification in panarthropods occurs during or before the formation of the blastoderm.

Insects

In most insects, the blastoderm is initially *syncytial*, with no cell membranes separating the nuclei (Anderson, 1973; Johannsen and Butt, 1941). In some insects, the primordial germ cells are the first cells to form, appearing at the posterior pole before the rest of the blastoderm is cellularized (Figure 1A1). Primordial germ cells that appear in this manner are called *pole cells*. Pole cells are most commonly seen in species of Diptera, Coleoptera, and Hymenoptera, although they have also been reported in Thysanoptera and Dermaptera (Table S1).

Pole cells were first reported in species of the midge *Chironomus* by Weismann (Weismann, 1863a), although Metchnikoff (1866) was the first to follow their migration into the gonad. In many insects, the cytoplasm at the posterior pole of the embryo is morphologically distinct from that of the rest of the embryo, often with visible granules (Hegner, 1914; Zissler, 1992). The close association of this granular cytoplasm with pole cells led researchers to speculate that the granular material contained germ line determinants (e.g. Hegner, 1909a; Zissler, 1992); we will describe experimental data testing this hypothesis below. It was long believed that pole cells gave rise to somatic cell types in addition to the germ cells (Anderson, 1962a; Anderson, 1962b; Poulson, 1947; Poulson, 1950). However, careful lineage tracing in the fruit fly *Drosophila melanogaster* showed that all pole cells are determined as germ cells, and any that become lost during their migration die (Underwood et al., 1980).

128 While the function of pole cells as primordial germ cells appears conserved, the
129 specific details of their formation can vary considerably across species. For example,
130 pole cells differ in which cleavage cycle they cellularize. Pole cells form during the ninth
131 mitotic cycle of *D. melanogaster* (Huettner, 1923) and during the sixth mitotic cycle in
132 the parasitic wasp *Nasonia vitripennis* (Buchta et al., 2013). To our knowledge, the
133 earliest observed formation of pole cells is during the third cleavage cycle in the midges
134 *Miastor metraloas* and *Chironomus sp*. (Hegner, 1914; Weismann, 1863b). The number
135 of pole cells formed is also variable: a single pole cell forms in embryos of the latter
136 midges (Hegner, 1914; Weismann, 1863b), whereas four appear in the fruit fly
137 *Bactrocera tryoni* (Anderson, 1962a) and sixteen appear in the beetle *Calligrapha*
138 *multipunctata* (Hegner, 1909b).
139 After the pole cells form, they enter the egg and migrate to the location of the
140 future embryonic gonad, which may be quite far from the posterior pole. Several
141 different migration pathways have been described. In *D. melanogaster* and many other
142 Diptera, the pole cells are carried into the egg during posterior midgut invagination, then
143 migrate through the epithelium of the posterior midgut to enter the abdominal mesoderm
144 and associate with the developing somatic gonad (Metschnikow, 1866; Sonnenblick,
145 1941). In Hymenoptera, pole cells are internalized with the rest of the mesoderm during
146 gastrulation (Gatenby, 1917). Yet another variation is observed in chrysomelid beetles
147 (Hegner, 1909a), where germ cells are internalized with the rest of the germ band as
148 the extraembryonic membranes fold over the germ band.
149
150 *Other arthropods*

151 Primordial germ cells are also specified early in Collembola, an early-diverging
152 branch of non-insect hexapods (Du et al., 2024) REF. Embryogenesis in these animals
153 begins with a period of holoblastic cleavage, and certain blastomeres on the basal (yolk)
154 side of the blastoderm can be recognized as likely primordial germ cells very early
155 (Jura, 1967; Klag, 1982). The cytoplasm of these germ cells contains granular material
156 visible by light and electron microscopy (Klag, 1982) that resembles the cytoplasm
157 containing germ line determinants observed in insects (Hegner, 1914; Zissler, 1992).
158 Rather than maintaining contact with the germ band as in insects, collembolan germ
159 cells migrate into the center of the yolk after their specification until eventually migrating
160 to associate with the abdominal mesoderm, from which the embryonic gonad develops
161 (Jura, 1967).
162 Among non-hexapod Pancrustacea, embryos of Copepoda, Amphipoda,
163 Decapoda, and some Cladocera undergo stereotypical holoblastic cleavage, allowing
164 precise tracking of cell lineages and the identification of a specific cleavage blastomere
165 as the progenitor of primordial germ cells (Gerberding et al., 2002; Hertzler, 2005; Kühn,
166 1913; McClendon, 1907). In amphipods and decapods, a putative germ plasm has been
167 identified in the freshly laid egg, termed the RNA-containing body (Gupta and Extavour,
168 2013) or intracellular body (Biffis et al., 2009; Chen et al., 2021). During cleavage, this
169 region of cytoplasm is inherited asymmetrically by the blastomeres that will give rise to
170 the primordial germ cells (Gupta and Extavour, 2013) (Figure 1A2).
171 In chelicerates, candidate primordial germ cells are first detectable with
172 molecular markers in the yolk of blastoderm-stage embryos in the mite *Tetranychus*
173 *urtichae* (Dearden et al., 2003). As the germ band forms, the primordial germ cells

174 associate with the posterior germ band near the primordia of the fourth walking legs. We
175 are not aware of any other chelicerate or myriapod species in which primordial germ cell
176 specification has been proposed to occur before gastrulation.
177
178 ***II.ii. Category 2: PGC specification at gastrulation***
179 After formation of the blastoderm, a portion of the blastoderm is specified as the
180 presumptive embryonic region, variably referred to as the *germ band*, *germ rudiment*,
181 *embryonic rudiment*, or *germ disc* depending on its initial form (Anderson, 1973;
182 Johannsen and Butt, 1941). The rest of the blastoderm differentiates into one or more
183 extraembryonic membranes (Johannsen and Butt, 1941). Soon after this specification,
184 *gastrulation* occurs, whereby some of the blastoderm cells are internalized and become
185 the mesoderm. In insects, mesoderm internalization primarily occurs along the ventral
186 midline of the germ band (Leptin, 2004; Roth, 2004). In other arthropods, mesoderm
187 may be internalized through a *blastopore* near the germ rudiment (Chaw et al., 2007;
188 Gerberding and Patel, 2004), or by epiboly (Edgar et al., 2015). The second category of
189 primordial germ cell differentiation occurs at or very close to the time of gastrulation.
190
191 *Insects*
192 Among insects, this timing of primordial germ cell specification is most common
193 among members of Hemiptera, Psocodea, and Lepidoptera, although it is also reported
194 in species of Dermaptera, Blattodea, Hymenoptera, Megaloptera, Strepsiptera,
195 Coleoptera, Mecoptera, and Diptera. We observed three different spatial patterns of
196 germ cell specification around the time of gastrulation: before germ band formation, at

197 the posterior of the germ band after its formation, or at the ventral midline of the
198 blastoderm just before gastrulation. In Hemiptera, Psocodea, and tenebrionid beetles
199 (Coleoptera), germ cells are first detectable at the ventral posterior region of the cellular
200 blastoderm in an invaginating structure called the *posterior pit* (Figure 1B) (Butt, 1949a;
201 Goss, 1952a; Mellanby, 1935a; Schröder, 2006; Shinji, 1919). These cells appear to
202 form on the side of the blastoderm epithelium facing the yolk, rather than on the outside
203 of the blastoderm like the pole cells described in Category 1. In Hemiptera and
204 Psocodea, the invagination continues into the egg towards the anterior, forming a
205 definitive germ band with germ cells at its posterior tip (Figure 1B1). Germ cells in
206 tenebrionid beetles move further inside the embryo once the amnion (ventral
207 extraembryonic membrane) has enclosed the entire ventral surface of the germ band,
208 similar to the internalization seen in Coleoptera that specify their germ cells as pole
209 cells. Following their formation, the germ cells migrate along the dorsal side of the germ
210 band toward the anterior, eventually settling into the somatic gonad in the abdominal
211 mesoderm.

212 In several species with this timing of germ cell specification, primordial germ cells
213 were first identified at the posterior tip of the germ band after it has detached from the
214 blastoderm [*e.g.*, *Periplaneta orientalis* (Blattodea) (Heymons, 1895), *Sialis mitsuhashii*
215 (Megaloptera) (Suzuki et al., 1981), *Nematus ribesii* (Hymenoptera) (Singh, 1967), and
216 Mecoptera (Suzuki, 1990)]. Early studies of tenebrionid beetles described this pattern of
217 germ cell differentiation as well (Rempel and Church, 1969; Ullmann, 1964). However,
218 more recent work using genetic markers suggests that primordial germ cells in at least
219 one such beetle, *Tribolium castaneum*, appear earlier, during the cellular blastoderm

220 stage in a posterior pit much like the Hemiptera (Schröder, 2006). This raises the
221 possibility that in the other tenebrionid species listed above, primordial germ cells may
222 likewise differentiate from the blastoderm, with the reported later appearance in the
223 germ band simply reflecting limitations in older detection methods.

224 In contrast to the posterior pit, germ cells differentiate along the ventral midline of
225 the blastoderm in Lepidoptera, separating from the blastoderm before gastrulation to lie
226 on the dorsal side of the presumptive germ band in the presumptive abdominal region
227 (Ando and Tanaka, 1980; Miya, 1958a; Presser and Rutschky, 1957; Tanaka, 1987;
228 Woodworth, 1889). Unlike when germ cells form at the posterior pit or at the posterior
229 tip of the germ band, in this case there does not appear to be long-range germ cell
230 migration, since the germ cells apparently form where the gonad eventually forms in the
231 abdomen.

232

233 *Other arthropods*

234 Among non-insect Pancrustacea, primordial germ cells are the first cells to be
235 internalized from the blastopore lip in Mysida (Manton, 1928), Anomopoda (Cannon,
236 1921), and the copepod orders Siphonostomatoida (McClendon, 1907), Harpacticoida
237 (Witschi, 1934), and Cyclopoida (Schimkewitsch, 1896). In Isopoda, primordial germ
238 cells differentiate from the mesendoderm soon after internalization (Figure 1B2)
239 (Goodrich, 1939a; Needham, 1942; Strömberg, 1965; Wolff, 2009). Thus, primordial
240 germ cell differentiation seems to be tied to gastrulation in these crustacean orders.

241 In chelicerates, primordial germ cells have been identified based on
242 morphological criteria at the blastopore in Opiliones (Figure 1B3) (Faussek, 1888;

243 Faussek, 1892; Holm, 1947) and Scorpiones (Brauer, 1894). A recent study using
244 molecular markers supported this interpretation in Opiliones (Gainett et al., 2022).
245 Putative primordial germ cells have also been identified at the blastopore in the
246 centipedes *Scolopendra cingulata* and *Strigamia maritima* (Myriapoda) (Green and
247 Akam, 2014; Heymons, 1901).

248 Tardigrade, germ cells are the first cells to enter the blastopore during
249 gastrulation, based on data from both morphological characteristics (Hejnol and
250 Schnabel, 2005) and molecular markers (Heikes et al., 2023) (Figure 1B4). Lineage
251 tracing in these embryos suggests that the germ cells are derived from both
252 blastomeres of the two-cell embryo (Gabriel et al., 2007; Hejnol and Schnabel, 2005).

253 Finally, onychophoran germ cells in different species emerge either at the
254 blastopore lip during gastrulation (Manton, 1949) or at the posterior end of the germ
255 band during elongation (Manton, 1949; Mayer and Tait, 2009; Sedgwick, 1887). We are
256 not aware of any studies using molecular markers to track germ cell formation in
257 Onychophora (Figure 1B5).

258

259 ***II.iii. Category 3: PGC specification from mesoderm***

260 After gastrulation, the germ band elongates and becomes segmented, and the
261 mesoderm becomes organized into pairs of somites in each segment. In most living
262 arthropods, each somite forms a small *coelom* enclosing a *coelomic cavity* (Anderson,
263 1973; Johannsen and Butt, 1941). The coelomic cavities are eventually lost as the
264 mesoderm differentiates into different cell types and reorganizes into the primordia of
265 different organ systems, including the somatic gonad (Anderson, 1973; Johannsen and

266 Butt, 1941). The final category of primordial germ cell differentiation timing
267 encompasses species in which primordial germ cells differentiate from the mesoderm at
268 this comparatively late stage of embryogenesis.
269
270 *Insects*
271 In insects, this category includes members of Archaeognatha, Zygentoma,
272 Orthoptera (Figure 1C1), Mantodea, Blattodea, Trichoptera, Coleoptera, and
273 Lepidoptera. Primordial germ cells may differentiate before formation of the coelomic
274 cavities [e.g., Orthoptera (Ewen-Campen et al., 2013a), Mantodea (Görg, 1959)], after
275 formation of the coelomic cavities [e.g., Archaeognatha (Larink, 1969), Zygentoma
276 (Woodland, 1957)], or after the coelomic cavities are no longer recognizable [e.g.,
277 Trichoptera (Miyakawa, 1974), *Odontotaenius disjunctus* (Coleoptera) (Krause, 1947),
278 *Apis mellifera* (Hymenoptera) (Dearden, 2006; Nelson, 1915)]. In this developmental
279 timing category, primordial germ cells are usually specified in the same abdominal
280 segments where the somatic gonad forms, so there is no long-range movement of
281 primordial germ cells after their specification.
282
283 *Other arthropods*
284 Differentiation of primordial germ cells from the mesoderm has also been
285 described in Leptostraca (Figure 1C2) (Manton, 1934), Anaspidacea (Hickman, 1936),
286 and Branchiopoda (Anderson, 1967; Mitsumoto and Makioka, 2002; Sudler, 1899).
287 However, data for stages of crustacean development late enough to include
288 differentiation of the trunk mesoderm from which primordial germ cells might arise are

289 sparse. This relative scarcity of early embryonic descriptions may be in part because
290 many crustaceans hatch as a nauplius, comprising only the head segments (Scholtz,
291 2000), and germ cells might not be specified until later larval stages, after formation of
292 the trunk. Many studies of naupliar and larval development in crustaceans are focused
293 on organogenesis of the gonad as a whole, without specific reference to the origin of
294 primordial germ cells (Cannon, 1924; Ikuta and Makioka, 1994; Mitsumoto and Makioka,
295 2002).

296 Classical studies on myriapods suggest a late differentiation of primordial germ
297 cells from the mesoderm in three of the four myriapod classes [Pauropoda (Tiegs,
298 1947), Symphyla (Tiegs, 1940), and Diplopoda (Dohle, 1964)], although only one
299 species was reported from each class in these studies. Germ cells in these classes
300 have not been investigated with molecular markers to our knowledge.

301 Among chelicerates, primordial germ cells differentiate from the mesoderm of the
302 opisthosomal segments in spiders (Figure 1C3) (Gough, 1902; Kautzsch, 1910;
303 Schimkewitsch, 1903). Data from molecular markers are consistent with this
304 observation (Schwager et al., 2015). Primordial germ cells are first detectable in the
305 mesoderm of the prosoma in pycnogonids (Alexeeva and Tamberg, 2021; Alexeeva et
306 al., 2018; Miyazaki and Makioka, 2012).

307

308 ***II.iv. Summary :evolutionary trends from late to early germ cell specification***

309 In his monograph on embryogenesis in Dermaptera and Orthoptera, Richard
310 Heymons (Heymons, 1895) proposed that “the formation of sex cells at the rear end of
311 the embryonic primordium, which has now been clearly demonstrated for

312 representatives of very different groups, is probably generalizable for all insects." Rather
313 than making broad statements such as this, we summarize the data regarding the
314 timing of germ cell specification in the context of currently accepted panarthropod
315 phylogeny (Ballesteros et al., 2022; Fernández et al., 2018; Giribet and Edgecombe,
316 2019; Misof et al., 2014; Schwentner et al., 2017) in Figures 2–3. This shows that in
317 early diverging euarthropod orders, primordial germ cells tend to be first observed later
318 in embryogenesis (Category 3). The timing of first germ cell appearance evolved to be
319 earlier in development in multiple lineages independently (Dermaptera, Condylognatha,
320 and Holometabola within insects; Copepoda, Malacostraca, and Cladocera within non-
321 hexapod Pancrustacea; Chilopoda within myriapods; and Opiliones, Scorpiones, and
322 mites within chelicerates). These data are consistent with the hypothesis that
323 differentiation from the mesoderm is the ancestral mode of primordial germ cell
324 specification in Euarthropoda, a hypothesis which we formally test below.
325 Conflicting interpretations in the literature about the timing of germ cell
326 specification have sometimes resulted from technical advances enabling identification of
327 germ cells at stages earlier than previously detected. For example, early work on the
328 silk moth *Bombyx mori* (Lepidoptera) reported primordial germ cells emerging from the
329 mesoderm of coelomic sacs in late embryogenesis (Category 3) (Toyama, 1902), while
330 later studies identified primordial germ cells on the posterior side of the ventral midline
331 of the early germ band (Category 2) (Miya, 1953). The onset of primordial germ cell
332 specification before gastrulation was later further supported by data from molecular
333 markers (Nakao, 1999; Nakao et al., 2006). Therefore, it is possible that species that
334 are currently classified in Category 3 may be reclassified in Categories 2 or 1 by future

work deploying molecular markers. This caveat notwithstanding, we note that molecular markers and functional genetic studies often corroborate late germ cell specification [see for example *Gryllus bimaculatus* (Orthoptera, Insecta) (Ewen-Campen et al., 2013a), *A. mellifera* (Hymenoptera, Insecta) (Dearden, 2006), *Parasteatoda tepidariorum* (Araneae, Chelicerata) (Schwager et al., 2015)]. We therefore do not assume that the hypothesis of ancestral late germ cell specification is an artifact of exclusive use of morphological identification methods.

Going forward, data from key early diverging lineages should be gathered to further test our hypothesis about the ancestral timing of primordial germ cell specification. Given the phylogenetic position of the Palaeoptera as basal branches of the hexapod tree, data about primordial germ cell specification in these orders would be especially useful to test our hypothesis with respect to hexapods, as would more information from the ametabolous Archaeognatha and Zygentoma or the non-insect hexapod Diplura. Similarly, data about primordial germ cell specification are missing from Oligostraca, the most basally branching clade of Pancrustacea, which includes Ostracoda and Branchiura.

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

358 detailed embryological studies in unexplored taxa, will therefore be needed to fully
359 understand the developmental context and evolutionary trends of germ cell specification
360 in these subphyla.
361
362 **III. Molecular and genetic mechanisms of germ cell specification in**
363 **panarthropods**
364 Beyond studying where and when germ cells first appear during arthropod
365 embryogenesis, researchers have sought to understand how they are specified. Germ
366 cell specification in animals has historically been classified into two mechanisms:
367 maternal inheritance and zygotic induction (Extavour and Akam, 2003; Nieuwkoop and
368 Sutasurya, 1981). In maternal inheritance, germ cell determinants accumulate in a
369 specific region of the oocyte cytoplasm, forming a germ plasm. Cells that inherit
370 sufficient germ plasm components after the embryo divides become primordial germ
371 cells. In embryos that undergo zygotic induction, all cells are in principle competent to
372 become germ cells at the beginning of embryogenesis. Certain cells later receive
373 signals from neighboring cells that induce them to take on germ cell identity. In the
374 following section, we will describe how historical observational data and perturbational
375 experimental approaches have offered increasingly robust evidence to distinguish
376 between these mechanisms in several panarthropod species.
377
378 ***III.i. Maternal inheritance***
379 In the early twentieth century, researchers speculated that the electron-dense,
380 membraneless granules frequently found in the cytoplasm of primordial germ cells were

381 responsible for their specification. Experiments in chrysomelid beetles provided early
382 evidence that the posterior cytoplasm of the oocyte or early embryo contains germ cell
383 determinants. Burning or puncturing the posterior poles of beetle eggs with needles to
384 damage or remove posterior cytoplasm reduced or eliminated primordial germ cells
385 (Hegner, 1908; Hegner, 1911), demonstrating that this cytoplasm played a causal, and
386 nor simply correlative, role in primordial germ cell specification. Studies in *D.*
387 *melanogaster* offered further evidence for the necessity of germ plasm. Removing or
388 destroying the posterior cytoplasm of *D. melanogaster* eggs via UV irradiation
389 demonstrated this region's requirement for germ cell formation (Geigy, 1931).
390 Subsequent centrifugation experiments in *D. melanogaster* embryos revealed that
391 shifting the granular structures in posterior cytoplasm away from the posterior pole
392 prevented germ cell formation or led to germ cells forming in a different part of the
393 embryo (Jazdowska-Zagrodzińska, 1966).
394 Experiments transplanting specific regions of cytoplasm between *D.*
395 *melanogaster* embryos showed that the contents of the posterior cytoplasm were
396 sufficient to specify germ cells. Transplanting posterior cytoplasm from undamaged *D.*
397 *melanogaster* embryos into sterile ones in which the posterior pole had been damaged
398 with UV-irradiation rescued fertility, while transplanting anterior cytoplasm did not
399 (Okada et al., 1974). Further transplantation experiments demonstrated that posterior
400 pole plasm introduced into the anterior of another embryo caused formation of ectopic
401 pole cells that gave rise to functional germ cells when transplanted again to the
402 posterior of a third, sterile embryo (Illmensee and Mahowald, 1974). Similar
403 experiments transferring the posterior cytoplasm from late-stage oocytes into embryos

404 revealed that the oocyte pole plasm could specify functional primordial germ cells
405 (Illmensee et al., 1976). This result revealed that the necessary germ cell determinants
406 in *D. melanogaster* are deposited during oogenesis. These foundational studies
407 demonstrated that maternally derived cytoplasmic granules are both necessary and
408 sufficient for germ cell formation in some species.

409 Contemporary studies testing for maternal inheritance often ask whether known
410 germ line gene products accumulate during late oogenesis and early embryogenesis in
411 the region where primordial germ cells first arise. For example, antibody staining or *in*
412 *situ* hybridization experiments to detect germ line molecules like Vasa protein or *nanos*
413 mRNA have suggested the presence of germ plasm in mosquitoes (Juhn and James,
414 2006; Juhn et al., 2008), scuttle flies (Wotton et al., 2014), ants (Khila and Abouheif,
415 2008; Lynch et al., 2011; Rafiqi et al., 2020), wasps (Kharel et al., 2024; Lynch et al.,
416 2011), weevils (Lynch et al., 2011), aphids (Chang et al., 2006; Chang et al., 2007;
417 Chen et al., 2009; Lin et al., 2014), amphipods (Gupta and Extavour, 2013), and prawns
418 (Chen et al., 2021). Transcriptomic analyses of different portions of embryos and
419 oocytes (Carter et al., 2015; Quan and Lynch, 2016; Yoon et al., 2019) have also
420 revealed asymmetric localization of molecules associated with primordial germ cell
421 specification.

422 However, expression data showing asymmetric localization of putative germ
423 plasm components are not sufficient to demonstrate that these molecules function in
424 primordial germ cell specification. For this, functional genetic techniques are needed to
425 manipulate gene activity and test the effect on germ plasm assembly and germ line
426 development. The function of germ plasm components in panarthropods has been most

427 thoroughly studied in *D. melanogaster* (Lehmann, 2016; Mahowald, 2001; Trcek and
428 Lehmann, 2019). The membraneless ribonucleoprotein granules at the posterior of *D.*
429 *melanogaster* oocytes and early embryos are nucleated by the protein Oskar, which is
430 both necessary and sufficient for germ plasm assembly (Ephrussi and Lehmann, 1992;
431 Kistler et al., 2018). Oskar-mediated localization of core germ line molecules such as
432 Vasa, Tudor, Aubergine, and *nanos* to germ granules is essential for primordial germ
433 cell specification and maintenance of germ cell identity during cell migration and gonad
434 formation in these fruit flies (Lehmann, 2016). The roles of these germ plasm molecules
435 in establishing the *D. melanogaster* germ line have been extensively reviewed
436 elsewhere (Lehmann, 2016; Mahowald, 2001).
437 Analysis of germ plasm composition and localization in species beyond *D.*
438 *melanogaster* has revealed both conserved and rapidly evolving features. Within the
439 *Drosophila* genus, germ granule size, morphology (Counce, 1963; Mahowald, 1968;
440 Rivard et al., 2025), and the relative proportions of different granule components vary
441 widely (Doyle et al., 2023). The wasp *N. vitripennis* also has a well-characterized germ
442 plasm in the form of a single large structure called the oosome (Bull, 1982). Like
443 *Drosophila, N. vitripennis* requires *oskar* to assemble a germ plasm containing many of
444 the same molecules (e.g., Vasa, Tudor, and *nanos*) (Lynch and Desplan, 2010; Lynch
445 et al., 2011). However, while *D. melanogaster* has many small germ granules
446 (Mahowald, 1962), the *N. vitripennis* oosome has a distinct protein organization and
447 transcript composition compared to that observed in *D. melanogaster* (Kemph et al.,
448 2025; Kharel et al., 2024; Lynch et al., 2011). In several crustacean species, the germ
449 plasm appears as a large, round granule in the cortex of embryos and contains

450 conserved germ plasm markers (Chen et al., 2021; Grattan et al., 2013; Gupta and
451 Extavour, 2013). Studying germ plasm in additional species will be needed to clarify
452 whether and how variation in germ plasm makeup and organization influences the post-
453 transcriptional regulation and function of germ line determinants.
454
455 ***III.ii. Zygotic induction***
456 Experimentally demonstrating inductive germ cell specification is more
457 technically challenging than detecting germ plasm accumulation. Consequently, most
458 studies have inferred or hypothesized zygotic induction based on absence of evidence
459 of germ plasm. For example, *in situ* hybridization experiments fail to reveal asymmetric
460 localization of putative germ line gene transcripts in oocytes and early embryos of
461 various species, including milkweed bugs (Ewen-Campen et al., 2013b), crickets
462 (Ewen-Campen et al., 2013a), honeybees (Dearden, 2006), mites (Dearden et al.,
463 2003), spiders (Schwager et al., 2015), centipedes (Green and Akam, 2014), and
464 tardigrades (Heikes et al., 2023). Instead, key germ line genes are expressed in a
465 subset of cells later in embryogenesis, coincident with reports of the first primordial
466 germ cells based on morphological criteria. However, we note that even in the well-
467 accepted case of mouse primordial germ cell induction (Saitou and Yamaji, 2010), it
468 remains formally possible that some unknown, and therefore untested, germ line
469 determinant, or factor that makes cells competent to respond to later germ line induction
470 signals, is asymmetrically localized and preferentially inherited by germ cells.
471 This caveat notwithstanding, there are multiple examples of experimental support
472 for induction rather than inheritance across panarthropods. Maternal knockdown of

473 putative germ plasm components in milkweed bugs and crickets reveals the
474 dispensability of maternal products of these genes for primordial germ cell formation
475 (Ewen-Campen et al., 2013b; Ewen-Campen et al., 2013a). Laser ablation of single
476 cells in two and four cell stage embryos of the tardigrade *Thuliana stephaniae* does not
477 prevent primordial germ cell specification, also suggesting that this species lacks a
478 cytoplasmic region with inherited germ line determinants (Hejnol and Schnabel, 2005).
479 Observations of germ cell formation in embryos with tissue duplications can be
480 interpreted as evidence against germ plasm and consistent with induction. For example,
481 in the drain fly *Clogmia albipunctata* and the flour beetle *T. castaneum*, genetic
482 manipulations producing double abdomen embryos lead to germ cell formation at both
483 anterior and posterior ends (Ansari et al., 2018; Yoon et al., 2019). Similarly, naturally
484 occurring “twin” embryos of the stick insect *Carausius morosus* results in duplicated
485 embryos fused along their anterior-posterior axis, that possess germ cells(Cavallin,
486 1971). In contrast, double abdomen embryos of *D. melanogaster* only produce germ
487 cells at the true posterior, since germ plasm is absent at the ectopic posterior end
488 (Lasko and Ashburner, 1990).
489 Among panarthropods, experiments in the cricket *G. bimaculatus* have provided
490 the most direct evidence for zygotic induction of primordial germ cells. Knockdown of
491 the mesoderm specification factor *twist* led to loss of primordial germ cells, suggesting
492 that mesoderm contributes to germ cell specification and/or maintenance (Ewen-
493 Campen et al., 2013a) and consistent with morphological evidence that orthopteran
494 primordial germ cells first arise from the embryonic mesoderm (Wheeler, 1893). The
495 BMP signaling pathway, known to induce germ cells in mice (Hopf et al., 2011; Lawson

496 et al., 1999; Ying and Zhao, 2001; Ying et al., 2000), is also required for cricket germ
497 cell specification. Specifically, BMP signaling and the downstream transcription factor
498 *Blimp-1* induce a subset of mesodermal cells to become primordial germ cells in
499 crickets (Donoughe et al., 2014; Nakamura and Extavour, 2016).
500 The shared use of both BMP and *Blimp-1* in mice and crickets may have
501 convergently evolved, or could suggest that this signaling mechanism is ancestral in
502 bilaterian inductive primordial germ cell specification (Lochab and Extavour, 2017).
503 Determining whether BMP and its downstream target genes are required for germ line
504 specification in more species will be necessary to distinguish between these
505 possibilities. Surprisingly few signaling pathways contribute to developmental fate
506 decisions in animals (Pires-daSilva and Sommer, 2003), increasing the likelihood of
507 evolutionary convergence, and limiting the number of candidate signaling pathways to
508 test for roles in primordial germ cell specification in different species.
509
510 ***III.iii. Intermediate and flexible strategies of germ cell specification***
511 Evidence from some animal species suggests that there may not be a strict
512 dichotomy between inheritance and induction (discussed by Seervai and Wessel, 2013).
513 These mechanisms may instead represent two ends of a spectrum, and they may not
514 be mutually exclusive. Below we discuss cases of panarthropod species for which data
515 suggests that their mechanisms of germ cell specification may fall somewhere along
516 this continuum.
517 In the silk moth *B. mori*, historical studies have generated conflicting evidence
518 about how germ cells are specified. Germ granules were not detected in early light

519 microscopy experiments, but germ cells failed to form after cauterization of the ventral
520 side of cleavage stage embryos, suggesting this region could contain germ cell
521 determinants (Miya, 1958a). Subsequent studies found that Vasa protein is not
522 asymmetrically localized before cellularization, again suggesting a lack of germ plasm
523 (Nakao et al., 2006). However, the mRNAs of one of four *B. mori nanos* homologs,
524 *nanosO*, localizes along the ventral midline of syncytial embryos immediately following
525 egg-laying, and is inherited by the primordial germ cells (Nakao et al., 2008).
526 Intriguingly, researchers found that maternal and zygotic *nanosO* act redundantly in
527 primordial germ cell specification; loss of one or the other reduces but does not
528 completely eliminate germ cells, whereas loss of both prevents germ cell formation
529 (Nakao and Takasu, 2019). The possibility that both inheritance and induction
530 mechanisms contribute to *B. mori* primordial germ cell specification suggests that this
531 species could offer insight into transitions in germ cell specification mechanisms within
532 Lepidoptera or in insects more broadly.
533 Although *D. melanogaster* is traditionally characterized as a species using a
534 germ plasm, zygotic signaling was recently suggested to contribute to germ cell
535 specification in this species (Colonnetta et al., 2022). Loss-of-function mutations in the
536 BMP family morphogen Dpp lead to partial loss of transcriptional quiescence and
537 mislocalization of germ plasm in the newly formed pole cells (Colonnetta et al., 2022).
538 Furthermore, loss of BMP signaling leads to activation of the normally suppressed
539 terminal signaling pathway in pole cells, which in turn disrupts their migration
540 (Colonnetta et al., 2022). These observations illustrate a universal conceptual challenge
541 in the field of developmental biology: distinguishing between, or even consistently

542 defining, cell type specification and maintenance. Although pole cells can form upon
543 disruption of BMP signaling, they do not contain the proper molecular components to
544 act as primordial germ cells. We suggest it may be most useful to define primordial
545 germ cell specification mechanisms as those that ensure both the physical formation of
546 germ cell precursors (including adoption of specific morphologies and activation of
547 specific gene regulatory states), and the competence of those cells to take the next
548 developmental steps toward joining the gonad. Under this definition, we would indeed
549 have to concede that both germ plasm and zygotic inductive mechanisms play roles in
550 *D. melanogaster* germ cell specification.

551 The ability to post-embryonically regenerate a germ line initially specified via
552 maternal inheritance has also been reported in multiple invertebrate species, including
553 the sea squirt *Ciona intestinalis* (Takamura et al., 2002), the earthworm *Enchytraeus*
554 *japonensis* (Tadokoro et al., 2006), and the marine worm *Capitella teleta* (Dannenberg
555 and Seaver, 2018) (see also Özpolat, 2024 for a review of this phenomenon in
556 Annelida)). The wasp *Pimpla turionellae* (Hymenoptera) has an oosome required for
557 germ cell specification: damaging this material results in no pole cells, which are the
558 primordial germ cells of this species (Achtelig and Krause, 1971). However, oosome-
559 deficient embryos lacking pole cells develop into larvae with germ cells in their gonad,
560 indicating that an additional mechanism must regenerate germ cells later in
561 development. Evidence of germ line regeneration has also been reported in the
562 amphipod *Parhyale hawaiensis*, which has a germ plasm that is asymmetrically
563 segregated to the *g* micromere and its lineage (Extavour, 2005; Gerberding et al., 2002;
564 Gupta and Extavour, 2013). Ablation of the *g* micromere eliminates primordial germ

565 cells in the embryo (Extavour, 2005), but the germ line is replaced post-embryonically
566 with germ cells that appear to derive from the mesoderm (Kaczmarczyk, 2014). The
567 authors of this study speculated that these replacement germ cells specifically derive
568 from cells of the somatic gonad(Kaczmarczyk, 2014) , reminiscent of a theory popular
569 for the first half of the 20th century, that primordial germ cells in most animals derived
570 from the inner epithelium of the somatic gonad (Everett, 1944). Species capable of
571 regenerating primordial germ cells present the opportunity to investigate the relative
572 contributions of and potential interactions between inheritance- and induction-based
573 mechanisms in a single species. Further, these species may represent living examples
574 of the intermediates that are hypothesized to exist in evolutionary transitions between
575 modes of germ line specification (Extavour, 2007).
576
577 ***III.iv. Summary: convergent evolution of panarthopod germ plasm***
578 The phylogenetic distribution of germ cell specification mechanisms across
579 panarthropods reveals a complex evolutionary history. Data for tardigrades,
580 chelicerates, and myriapods are consistent with induction as the ancestral mechanism
581 of panarthropod primordial germ cell specification. Pancrustacea are the most heavily
582 represented group in our dataset. The late origin of primordial germ cells in several
583 species suggests induction may be widely used, but there appear to be independent
584 origins of germ plasm in Malacostraca and Branchiopoda. The use of induction and
585 germ plasm in different decapod species suggests that further sampling could reveal
586 patterns in the evolution of germ cell formation within this order (Biffis et al., 2009; Chen
587 et al., 2021; Terao, 1929). However, functional experiments and broader taxonomic

588 sampling will be necessary to confirm mechanisms and reconstruct their evolutionary
589 trajectories across Panarthropoda as a whole.

590 Reminiscent of the broader pattern we previously reported across animals
591 (Extavour, 2007; Extavour and Akam, 2003; Whittle and Extavour, 2017), in some insect
592 orders we noted that early diverging lineages deployed induction, while later lineages
593 appeared to use germ plasm. This pattern is well exemplified in Coleoptera, (Kobayashi
594 et al., 2006; Komatsu and Kobayashi, 2012; Krause, 1947; Niikura et al., 2017;
595 Schröder, 2006; Ullmann, 1964), with pole cells forming only in Chrysomeloidea and
596 Curculionidae (Brauer, 1925; Hegner, 1908; Lynch et al., 2011). In addition, in
597 Carabidae, a basally branching beetle family that lacks pole cells, *oskar* orthologs have
598 been identified, decoupling the presence of *oskar* from germ plasm in beetles (Blondel
599 et al., 2021; Kobayashi et al., 2013). These observations raise the possibility that germ
600 plasm may not be ancestral to Coleoptera. When considered alongside the absence of
601 germ plasm in most Lepidoptera and the observation of late-arising, Category 3 germ
602 cells in Trichoptera (Miyakawa, 1974) and Category 2 germ cells in Mecoptera (Suzuki,
603 1990), these patterns are consistent with a novel hypothesis in which germ plasm
604 evolved independently multiple times across insects, rather than only once in the
605 Holometabola as we and others have previously proposed (Ewen-Campen et al., 2012;
606 Ewen-Campen et al., 2013a; Lynch et al., 2011). Our previous work had suggested the
607 independent evolution of germ plasm at the phylum level (Extavour and Akam, 2003),
608 but this new hypothesis is the first, to our knowledge, to suggest such widespread
609 convergent evolution of maternal inheritance-based germ cell specification within a
610 phylum.

611 To test this hypothesis, we performed ancestral character reconstruction using
612 both maximum parsimony and maximum likelihood approaches. For this analysis, we
613 designated species as utilizing inheritance or induction based on experimental evidence
614 when available or inferred from observational data describing the timing and location of
615 germ cell specification (Table S1). The results of the parsimony analysis inferred that
616 the ancestral panarthropod specification mode was induction, with a minimum of 24
617 changes over the history of the panarthropods (Figure S1; Table S3). When considering
618 all equally parsimonious character histories, changes from induction to maternal
619 inheritance (min = 18, max = 23, average = 20.5) were the most common, but reverse
620 transitions were also inferred (min = 2, max = 7, average = 4.5). At a minimum,
621 reversions from induction to maternal inheritance were inferred in the honeybee *A.*
622 *mellifera* (Hymenoptera) and drain fly *C. albipunctata* (Diptera). We note that all
623 maximum parsimony reconstructions assign induction as the ancestral state of
624 Holometabola (node 11), which requires many fewer total transitions within
625 Holometabola (N = 12) than would be required if germ plasm evolved a single time in
626 the last common ancestor of Holometabola (N = 20).
627 The marginal ancestral reconstructions of our maximum likelihood approach are
628 consistent with the parsimony analysis described above (see Table S3 for a
629 comparison), again providing strong statistical support for induction as the ancestral
630 specification mechanism in Panarthropoda, Arthropoda, and Insecta, with multiple
631 independent instances of germ plasm evolution and reversion to induction (Figure S2;
632 see Table S7 for marginal probabilities). We further find strong evidence that induction
633 was the ancestral specification mechanism in Holometabola (probability 80.6%),

634 followed by independent transitions to germ plasm in the common ancestors of Diptera
635 and Mecoptera (or independently within Mecoptera, Siphonaptera, and Diptera), derived
636 lineages of Lepidoptera, in the common ancestor of the families Chrysomelidae and
637 Curculionidae (within Coleoptera), and in the common ancestor of Hymenoptera.
638 Outside of Holometabola, we infer independent transitions to germ plasm in thrips,
639 aphids, earwigs, Collembola (which may have undergone multiple independent
640 transitions within the class), Decapoda, the last common ancestor of Hyalidae and
641 Talitridae (order Amphipoda), and in the chelicerate *Tetranychus urticae*.
642

643 **IV. Evolution of panarthropod germ cell specification**

644 In the following section, we discuss the developmental and genetic factors that might
645 contribute to evolutionary transitions in germ cell specification across Panarthropoda.
646

647 ***IV.i. The relationship between germ cells and somatic germ layers***

648 In arthropods with late formation of primordial germ cells (Category 3), primordial
649 germ cells appear to differentiate directly from the mesoderm (Evans, 1901; Hickman,
650 1936; Nelson, 1915). Consistent with this hypothesis, abolishing mesoderm prevents
651 germ cell specification in the cricket *G. bimaculatus* (Ewen-Campen et al., 2013a).
652 Germ cell differentiation occurs concurrently with the differentiation of many other
653 mesodermal cell types, including the somatic gonad, musculature, heart, and fat body.
654 We suggest that the types of mechanisms that specify germ cells are not distinct in
655 principle from those specifying any other mesodermal cell type in these animals.

656 The germ layer origin of germ cells is less clear in arthropods whose germ cells
657 appear before or very close to the time of gastrulation (Category 2). For example,
658 primordial germ cells in many crustaceans are described as being the first to enter the
659 blastopore during gastrulation (e.g. Cannon, 1921; Goodrich, 1939b; Manton, 1928),
660 and usually considered by the authors to be derived from the mesendoderm. In
661 contrast, germ cells in Category 2 insects arise before gastrulation (Mellanby, 1935b;
662 Ullmann, 1964), consistent with the hypothesis that germ cells are distinct from the
663 mesoderm and may not derive from any germ layer.
664 In crustaceans in which a putative germ plasm has been identified, the cleavage
665 pattern is stereotypical, and cell lineages are available, the germ cell lineage is sister to
666 the mesoderm or the mesendoderm [Amphipoda (Extavour, 2005; Gerberding et al.,
667 2002; Scholtz and Wolff, 2002; Wolff and Scholtz, 2002), Euphausiacea (Alwes and
668 Scholtz, 2014), Decapoda (Hertzler, 2005)], but never to the ectoderm. In addition,
669 removing the putative germ plasm from embryos of the amphipod *P. hawaiensis* makes
670 the *g* micromere morphologically indistinguishable from the other micromeres, which
671 give rise to mesoderm or endoderm in unperturbed embryos (Gupta and Extavour,
672 2013). This result suggests that in the absence of germ cell determinants, the *g*
673 micromere, normally the germ line progenitor, could instead adopt mesendodermal fate.
674 This hypothesis has not been directly tested in this amphipod because gastrulation fails
675 after ablation of the putative germ plasm (Gupta and Extavour, 2013). Together, these
676 data suggest a close association between the germ cell and mesendodermal lineages in
677 these crustaceans. This relationship is apparent beyond Pancrustacea, since germ cells
678 induced late in embryogenesis arise from the mesoderm (Category 3). We thus

679 speculate that germ cells in panarthropods may be ancestrally derived from the
680 mesodermal lineage. We further speculate that inductive germ cell specification is
681 ancestral to panarthropods, and present a formal test of this hypothesis below.
682
683 ***IV.ii. Evolutionary shifts in the spatial and temporal origin of primordial germ cells***
684 We have seen significant variation in the spatial and temporal origin of primordial
685 germ cells across panarthropods (Figures 2-3; Table S1). However, regardless of when
686 primordial germ cells first appear, we find a common stage across many panarthropods
687 in which primordial germ cells are situated in bilateral segmental clusters across
688 multiple trunk segments. Soon after this stage, the segmental clusters on each side fuse
689 together into bilaterally symmetrical genital ridges, corresponding to the primordial
690 embryonic gonad (Miya, 1958b; Nelsen, 1934; Seidel, 1924; Ullmann, 1964).
691 Most arthropods undergo “short germ” development, wherein the blastoderm
692 tissue generates only anterior segments, with posterior segments being added
693 sequentially to the posterior end of the germ band later in development. In contrast, the
694 “long germ” mode of development seen in *D. melanogaster*, wherein the blastoderm
695 tissue immediately generates all body segments, is an evolutionary novelty found only
696 in some insects (Clark et al., 2019; Krause, 1939). In Category 1 or 2 arthropods with
697 short germ development, germ cells are often specified before the formation of the
698 segments that will include the future embryonic gonad. In Category 2 insects, germ cells
699 often associate with the posterior end of the germ band and then migrate toward the
700 anterior as the germ band elongates (Goss, 1952b; Hegner, 1909b; Heming and
701 Huebner, 1994; Paz, 1958; Suzuki, 1990; Ullmann, 1964). This migratory path is so

common among insects that Johanssen and Butt included it in their description of embryogenesis in a stereotypical insect (Johannsen and Butt, 1941).

The association of germ cells with the posterior end of the embryo may have had other evolutionary consequences in insects. Many insects, both long and short germ, specify the body plan early in development via maternal deposition of axial patterning determinants, independent of the mechanism of germ cell specification (reviewed in Lynch, 2019; Rosenberg et al., 2009). For example, the short germ grasshopper *Schistocerca americana* exhibits asymmetric localization of the posterior patterning determinant *nanos* in the absence of a germ plasm (Lall et al., 2003). However, insects with germ plasm that includes axial patterning determinants, like *D. melanogaster*, often exhibit long germ development. Based on these observations, we employed phylogenetic logistic regression and Pagel's test of evolutionary rate covariation (Pagel, 1994) to test the hypothesis that the acquisition of long germ band development influences the evolutionary rate of germ cell mechanism transitions or vice versa, and found statistically significant evidence of this association using both methods (Figure S3; Figure S4). All Pagel covariation models assuming coupled evolutionary rates among these two characters are significantly better fits to the data than the null model of independent evolution (likelihood ratio test p-value $< 1e{-}3$; Figure S4; Table S8). We note that the bidirectional and GB-dependent models perform similarly to the GC-dependent model, suggesting both that long germ band development may drive the evolution of germ plasm, and that germ plasm may drive transitions to long germ band development.

724 We further hypothesize that a spatial shift in germ cell specification to the
725 posterior end of the embryo in Category 2 insects created a condition in which germ line
726 determinants are localized to the same embryonic region as posterior patterning
727 determinants (Figure 4B). Subsequently, germ line determinants may have hitchhiked
728 onto the asymmetric localization machinery, coupling germ cell specification to posterior
729 patterning and thus promoting the evolution of germ plasm (Figure 4C). Under this
730 hypothesis, the germ plasm of *D. melanogaster* would represent the final stage of this
731 evolutionary scenario, as its germ granules include both germ line and abdominal
732 patterning determinants (Ephrussi and Lehmann, 1992; Trcek et al., 2015; Wang and
733 Lehmann, 1991).

734 *Nasonia vitripennis* is an intriguing case to study the relationship between
735 anteroposterior patterning and germ cell specification. The posterior cytoplasm of this
736 wasp has two populations of *nanos*: one that is incorporated into the oosome and
737 eventually into pole cells, and another that functions in axial patterning (Lynch and
738 Desplan, 2010). *nanos* localization to the oocyte posterior in this wasp depends on
739 actin-dependent anchoring (Olesnicky and Desplan, 2007), just as in *D. melanogaster*
740 (Forrest and Gavis, 2003), suggesting a conserved localization mechanism between
741 these two species. We speculate that *N. vitripennis* represents an evolutionary
742 intermediate between species where anteroposterior patterning is entirely independent
743 of germ cell formation (Figure 4A) and those where these two processes are fully linked
744 (Figure 4C). Our evolutionary hypothesis can be tested further by examining the
745 mechanisms of localization of posterior patterning determinants and germ line

746 determinants in more insect species to understand how existing cytoskeletal machinery
747 for one developmental process may have been coopted for use in another.
748
749 ***IV.iii. Potential gene regulatory basis of evolutionary shifts in the molecular***
750 ***mechanism of primordial germ cell specification***
751 Beyond the timing and position of germ cell origin, evolutionary changes in the
752 localization of gene products and timing of gene expression may also underlie
753 transitions in the mechanism of specification (Extavour, 2007; Srouji and Extavour,
754 2010). Many of the genes important for specifying germ cells are conserved across
755 metazoans (Ewen-Campen et al., 2010), but they exhibit distinct temporal and spatial
756 expression patterns across species. In species that use inheritance, the germ line gene
757 products are expressed during oogenesis and persist in the early embryonic cytoplasm
758 to be inherited by the primordial germ cells. In species that induce germ cells, these
759 genes are either not expressed in oocytes or the gene products are not asymmetrically
760 localized so they are not preferentially inherited by particular cells. As we have
761 suggested previously (Extavour, 2007; Srouji and Extavour, 2010), relatively simple
762 regulatory modifications affecting post-transcriptional regulation, the cytoskeleton, or
763 RNA or protein localization could cause germ line gene product retention in oocytes,
764 thereby enabling the transition from induction to inheritance.
765 The insect-specific gene *oskar* offers a potential example of gene network
766 evolution driving shifts in germ cell specification mechanism. Since *oskar* is likely a
767 nucleator of germ plasm in multiple insects (Kemph and Lynch, 2022; Lynch et al.,
768 2011) and has been lost multiple times since its origin prior to the divergence of

769 Pterogyta from Ametabola (Blondel et al., 2021), we hypothesized that *oskar* gene
770 presence might be correlated with germ line specification mode in a given taxon (Figure
771 S3). A related hypothesis, that the evolutionary advent of *oskar* gave rise to
772 holometabolous germ plasm, was previously proposed based on the co-occurrence of
773 *oskar* transcript and germ plasm in *N. vitripennis* and *D. melanogaster*, as well as the
774 absence of both *oskar* homologs and f germ plasm in *T. castaneum*, *A. mellifera*, *C.*
775 *albipunctata*, and most Lepidoptera (Lynch et al., 2011). The results of applying
776 phylogenetic logistic regression to this hypothesis (Figure S3; Table S9) suggest a
777 strong and statistically significant association between *oskar* presence and germ-plasm
778 mode (coefficient = 2.5827, adjusted odds-ratio: = 13.28, p-value = 0.00059) and no
779 association of germ-plasm mode with BUSCO completeness (coefficient = 0.228, p-
780 value = 0.368). The association between *oskar* presence and germ-plasm mode
781 remains strong and statistically significant even after removing 24 within-family
782 substitutions made when neither genomic nor transcriptomic data were available for a
783 species (coefficient = 2.56, adjusted odds ratio = 12.929, p-value = 0.00026). We
784 emphasize that this observed association does not imply that *oskar* is necessary or
785 sufficient for germ plasm formation; indeed, there are insect species without *oskar* that
786 nevertheless have germ plasm and species with *oskar* that lack germ plasm (Figure
787 S3), as we discuss further below.
788 In contrast to prior hypotheses from our group and others of a single origin of
789 germ plasm at the base of the Holometabola (Ewen-Campen et al., 2012; Ewen-
790 Campen et al., 2013a; Kemph and Lynch, 2022; Lynch et al., 2011), our current
791 likelihood and parsimony reconstruction of induction as ancestral to Holometabola

792 (Figure 5; Figures S1-S2) implies that *oskar* has been independently recruited as a
793 germ plasm nucleator at least twice in Hymenoptera (Lynch et al., 2011) and Diptera
794 (Ephrussi and Lehmann, 1992). The strong association between *oskar* presence and
795 germ plasm (Figure S3) implies that it may have been co-opted for germ plasm
796 nucleation even more times across insects. We note that such “gene reuse” is a
797 common feature of convergent evolution across multiple biological processes (Chan et
798 al., 2010; Martin and Orgogozo, 2013; Morris, 2009; Reed et al., 2011; Satterlee et al.,
799 2024). Since *oskar* is co-expressed with germ plasm components in nervous systems of
800 hemimetabolous and holometabolous insects (Ewen-Campen et al., 2012; Kulkarni et
801 al., 2023; Xu et al., 2013), we posit that independent acquisitions of germ plasm in
802 distinct lineages may have proceeded via parallel cooption of a pre-existing gene
803 interaction network. Future studies should aim to determine whether each independent
804 origin of germ plasm in the Holometabola arose by co-option of *oskar* to the germ line,
805 or by *oskar*-independent mechanisms of germ plasm assembly.
806 The results of our analysis (Figure S3; Table S9) suggest that *oskar*’s functional
807 evolution may have contributed to a transition from induction to inheritance, but
808 precisely when and how *oskar* acquired a germ line role remains unclear. Morphological
809 data suggest that thrips use germ plasm (Haga, 1985; Heming, 1979), and *oskar*
810 homologs have been found in this order (Blondel et al., 2021). If *oskar* plays a role in
811 thrips germ cell specification, it could support the entirely novel hypothesis that *oskar*
812 evolved its germ line determinant properties before the evolution of insect
813 metamorphosis.
814

IV.v. The case for novel panarthropod germ plasm nucleators

Recruitment of *oskar* to the germ line is not the only mechanism explaining the evolution of germ plasm in arthropods. The hemimetabolous pea aphid *Acyrthosiphon pisum* lacks *oskar* but has a germ plasm containing Vasa and Nanos (Chang et al., 2006; Chen et al., 2009; Lin et al., 2014), which are asymmetrically localized by unknown mechanisms. Vasa is also asymmetrically localized to a putative germ plasm in amphipod and decapod species (Chen et al., 2021; Gupta and Extavour, 2013), which must also have evolved via distinct mechanisms since *oskar* is an insect-specific gene (Blondel et al., 2021). Germ plasm nucleators have convergently evolved in diverse animal phyla (Aoki et al., 2016; Bontems et al., 2009; Kulkarni and Extavour, 2017; Lee et al., 2020; Marlow and Mullins, 2008; Scholl et al., 2024). Similarly, we posit that germ plasm nucleators other than *oskar* must also exist across the panarthropods, awaiting discovery.

V. Concluding thoughts

By reinterpreting the meticulous work of generations of classical embryologists through the lens of modern developmental genetics and an improved panarthropod phylogeny, we have generated novel, testable evolutionary hypotheses about the mechanisms that specify the germ line, with implications for the genetic basis of convergence in evolution that extend beyond this superphylum. The advent of new genetic tools in an increasing number of panarthropod model species place us in an exciting era to tackle age-old questions about the evolution of panarthropod germ cell

specification, as well as the impact of these mechanisms of genome evolution and body 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 25384e7.

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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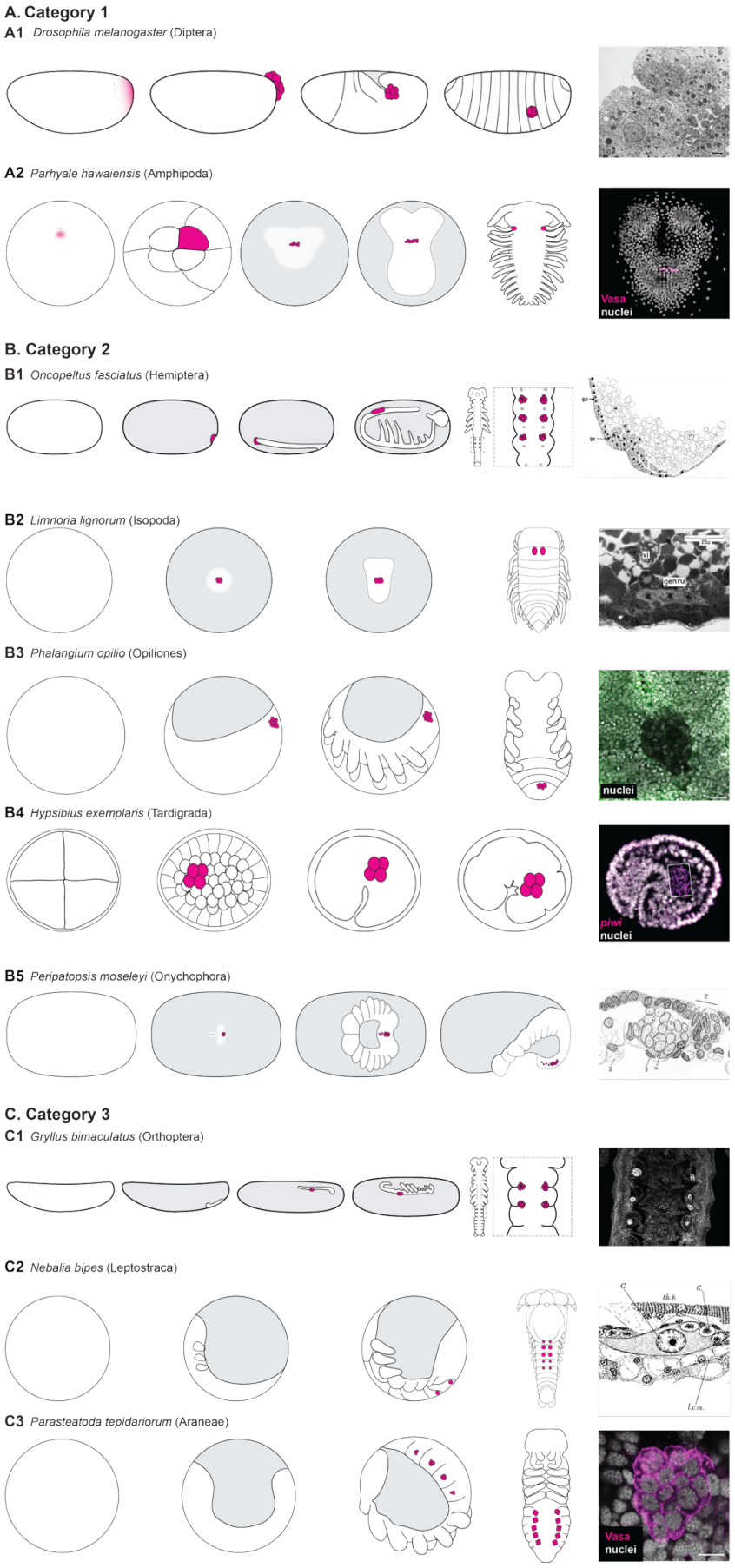
Conflicts of Interest

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)
B3 Phalangium opilio (Opiliones)
nuclei
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

867 **Figure 1 (previous page). Primordial germ cell origin in the context of panarthropod**
868 **embryogenesis.** In all schematics, magenta represents the approximate location of germ plasm or
869 primordial germ cells; light gray represents yolk or extraembryonic tissue.
870 (**A1**) Schematic: In the fruit fly *Drosophila melanogaster*, germ plasm accumulates at the posterior of
871 syncytial embryos and is inherited by pole cells that migrate to the ultimate site of the embryonic gonad
872 (Illmensee and Mahowald, 1974). Data: TEM micrograph displays pole cells budding in *D. melanogaster*
873 embryos. The pole cell nuclei (N) have characteristically diffuse chromatin, and polar granules (P) are
874 distributed throughout the cytoplasm. Image reproduced with permission from (Mahowald, 1968).
875 (**A2**) Schematic: In the amphipod *Parhyale hawaiensis*, germ plasm accumulates in a region of the
876 embryo called the RNA-containing body, which is inherited by the *g* micromere in the 8-cell stage
877 (Extavour, 2005; Gupta and Extavour, 2013). All descendants of the *g* micromere are primordial germ
878 cells. Data: Primordial germ cells can be visualized by immunohistochemistry with a cross-reactive
879 antibody raised against recombinant Vasa protein from both *D. melanogaster* and the grasshopper
880 *Schistocerca americana* (Chang et al., 2002).
881 (**B1**) Schematic: In the milkweed bug *Oncopeltus fasciatus*, primordial germ cells first appear in the
882 ventral posterior blastoderm in an invaginating structure called the posterior pit (Ewen-Campen et al.,
883 2013b). Primordial germ cells remain for some time at the posterior tip of the germ band as it invaginates
884 into the yolk. The germ cells migrate along the germ band toward the anterior and settle in segments A4–
885 A6. Data: Light micrograph of sectioned *O. fasciatus* embryos shows germ cells in the posterior pit in the
886 late blastoderm stage. Image reproduced with permission from (Butt, 1949b).
887 (**B2**) Schematic: In the isopod *Limnoria lignorum*, primordial germ cells are first identifiable among the
888 mesendodermal mass just after the beginning of gastrulation (Strömberg, 1968). Primordial germ cells
889 remain associated with the mesoderm throughout development. Data: Light micrograph of a sectioned *L.*
890 *lignorum* embryo shows primordial germ cells among the mesoderm in the extended germ band stage,
891 distinguished by their lighter-staining cytoplasm and larger nuclei. Image reproduced with permission from
892 (Strömberg, 1968).
893 (**B3**) Schematic: In the harvestman *Phalangium opilio*, primordial germ cells are first identifiable at the
894 posterior end of the germ band soon after the germ band forms (Faussek, 1888; Gainett et al., 2022).
895 Primordial germ cells remain at the posterior end of the germ band at least until the fourth opisthosomal
896 segment is formed, after which the germ cells can be found in that segment. Data: Primordial germ cells
897 can be identified by staining with the nucleic acid dye Hoechst 33342; germ cell chromatin is more diffuse
898 compared with that of surrounding cells. Image kindly provided by Prashant Sharma (University of
899 Wisconsin, Madison).
900 (**B4**) Schematic: In the tardigrade *Hypsibius exemplaris*, primordial germ cells are first identifiable in the
901 32-cell stage and are the first cells to be internalized during gastrulation. Data: Primordial germ cells can
902 be visualized by *in situ* hybridization against *He-vasa*. Image kindly provided by Kira Heikes (Duke
903 University).
904 (**B5**) Schematic: In the velvet worm *Peripatopsis moseleyi*, primordial germ cells are first identifiable near
905 the blastopore at the beginning of gastrulation. As the embryo elongates and more segments form, the
906 primordial germ cells migrate to more anterior segments. Data: Light micrograph of a *P. moseleyi* embryo
907 shows primordial germ cells soon after their internalization during gastrulation, distinguished by their
908 lighter-staining nuclei and cytoplasm. Image reproduced with permission from (Manton, 1949).
909 (**C1**) Schematic: In the cricket *Gryllus bimaculatus*, primordial germ cells are first detectable among the
910 mesoderm in segments A2–A4 in embryonic stage 6, after most abdominal segments have been
911 specified (Ewen-Campen et al., 2013a). Data: Primordial germ cells can be visualized by
912 immunohistochemistry with an antibody against *Gb*-Vasa.
913 (**C2**) Schematic: In the leptostracan *Nebalia bipes*, primordial germ cells are first identifiable in the
914 mesoderm in the thorax and abdomen during the extended germ band stage (Manton, 1934). Data: Light
915 micrograph of a sectioned *N. bipes* embryo shows primordial germ cells among the mesoderm in the
916 extended germ band, distinguished by their lighter-staining cytoplasm and larger nuclei. Image
917 reproduced with permission from (Manton, 1934).
918 (**C3**) Schematic: In the house spider *Parasteatoda tepidariorum*, primordial germ cells are first identifiable
919 in segmental clusters in the opisthosomal mesoderm after all segments have formed (Schwager et al.,
920 2015). Data: Primordial germ cells can be visualized by immunohistochemistry using an antibody raised
921 against *Pt*-Vasa.

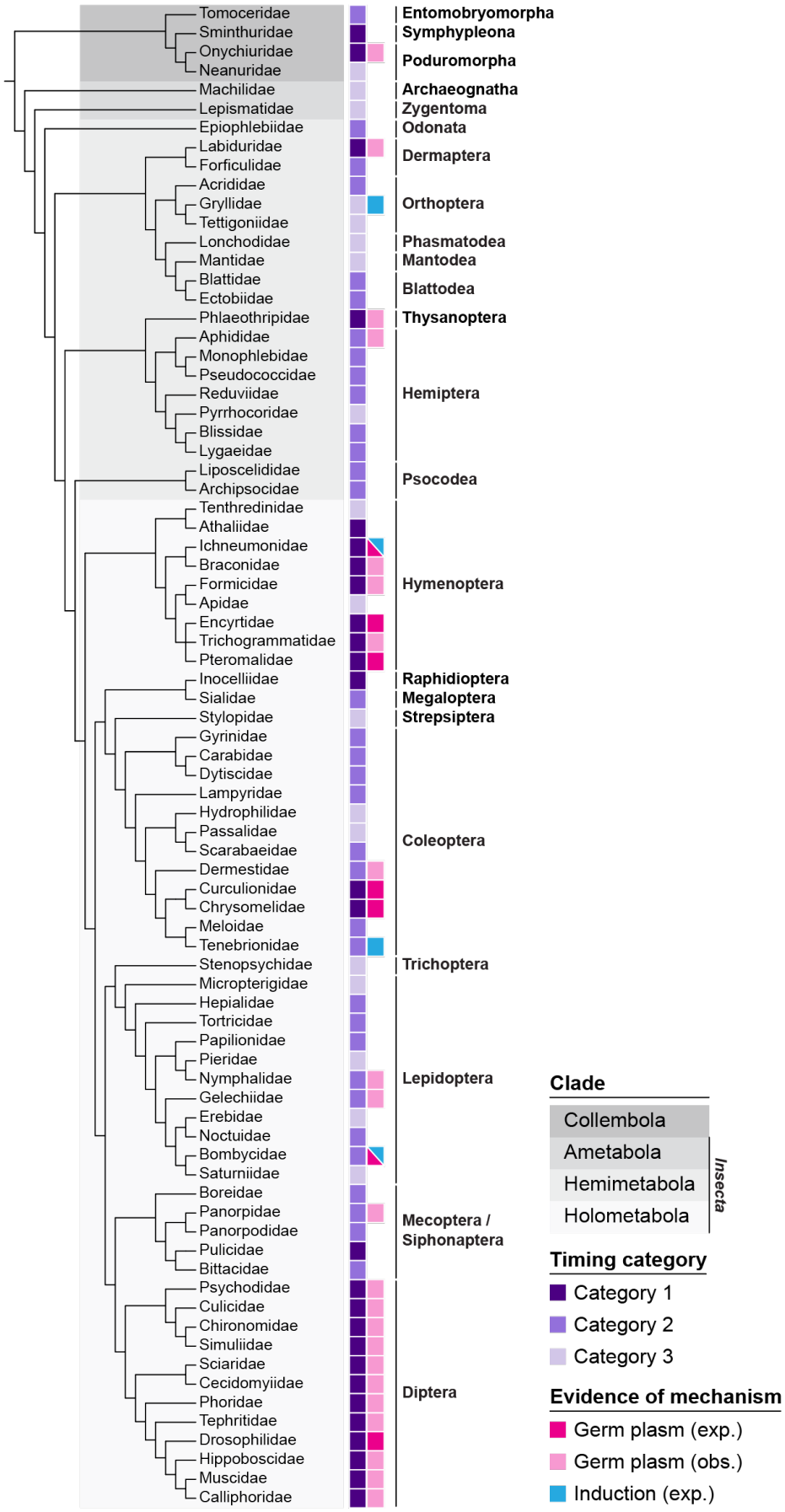
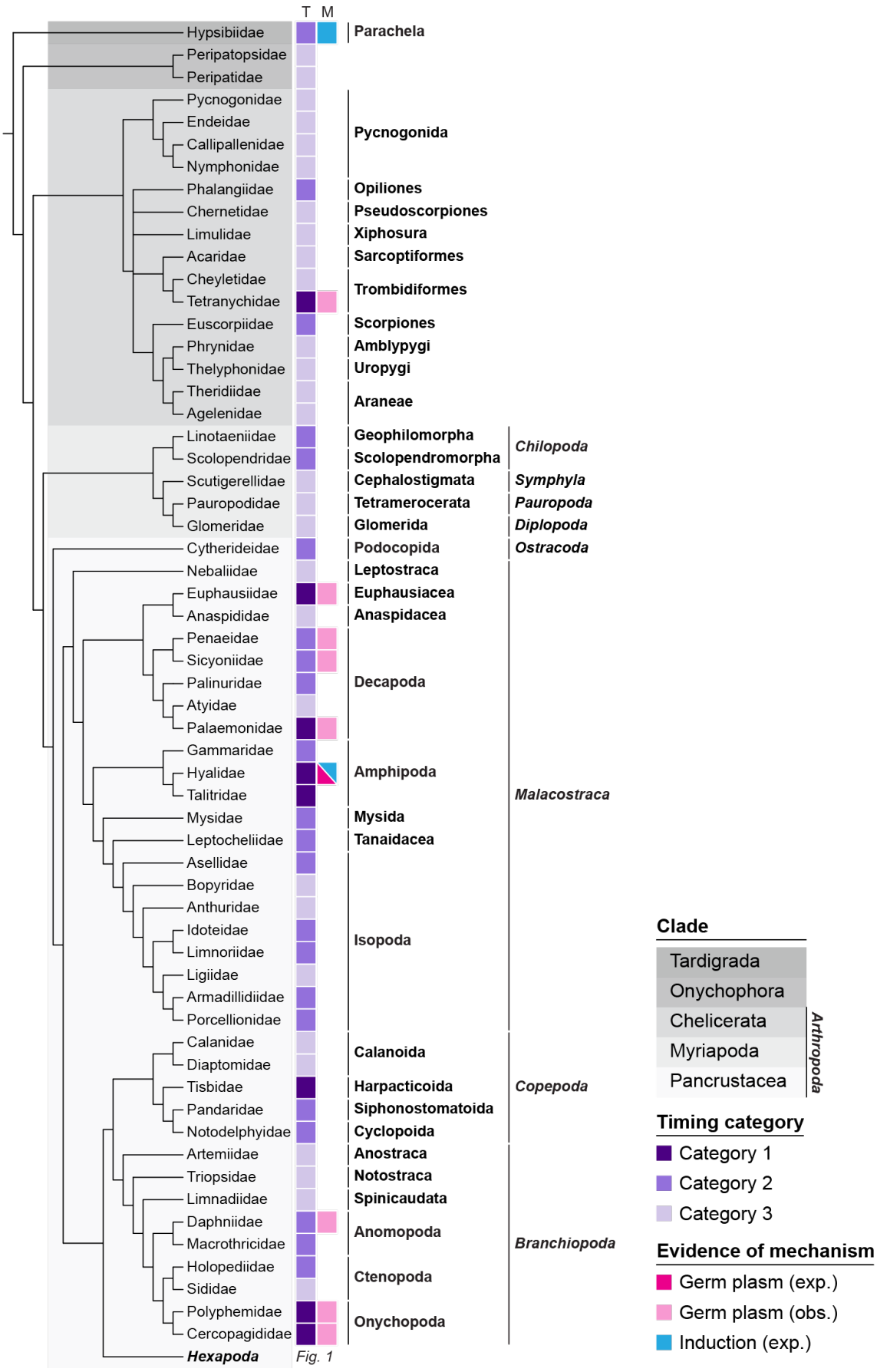

Tomoceridae
Sminthuridae
Onychiuridae
Neanuridae
Machilidae
Lepismatidae
Epiophlebiidae
Labiduridae
Forficulidae
Acrididae
Gryllidae
Tettigoniidae
Lonchodidae
Mantidae
Blattidae
Ectobiidae
Phlaeothripidae
Aphididae
Monophlebidae
Pseudococcidae
Reduviidae
Pyrrhocoridae
Blissidae
Lygaeidae
Liposcelididae
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.)

Figure 2 (previous page). Germ cell specification across Hexapoda. Phylogeny of studied hexapod lineages marking the origin category and the mechanism of primordial germ cell specification. Phylogenetic relationships in the cladogram are derived from published relationships described in the Supplementary Material (Misof et al., 2014)and branch lengths are not to scale. Data on the timing of germ cell specification in the first column. Dark purple = Category 1 (cleavage/blastoderm), medium purple = Category 2 (gastrulation), light purple = Category 3 (mesoderm differentiation). Data about the mechanism of germ cell specification are found 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 are described in more detail in Table S1.


T
M
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
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
Fig. 1
Clade
Tardigrada
Onychophora
Chelicerata
Myriapoda
Pancrustacea
Arthropoda
Timing category
Category 1
Category 2
Category 3
Evidence of mechanism
Germ plasm (exp.)
Germ plasm (obs.)
Induction (exp.)

933 **Figure 3 (previous page). Panarthropod germ cell specification outside of Hexapoda.** Phylogeny of
934 studied non-hexapod panarthropod lineages marking the origin category and mechanism of primordial
935 germ cell specification. Phylogenetic relationships are derived from published relationships described in
936 the Supplementary Material (Misof et al., 2014)and branch lengths are not to scale. Colors are the
937 same as in Figure 2. Data are described in more detail in Table S1.

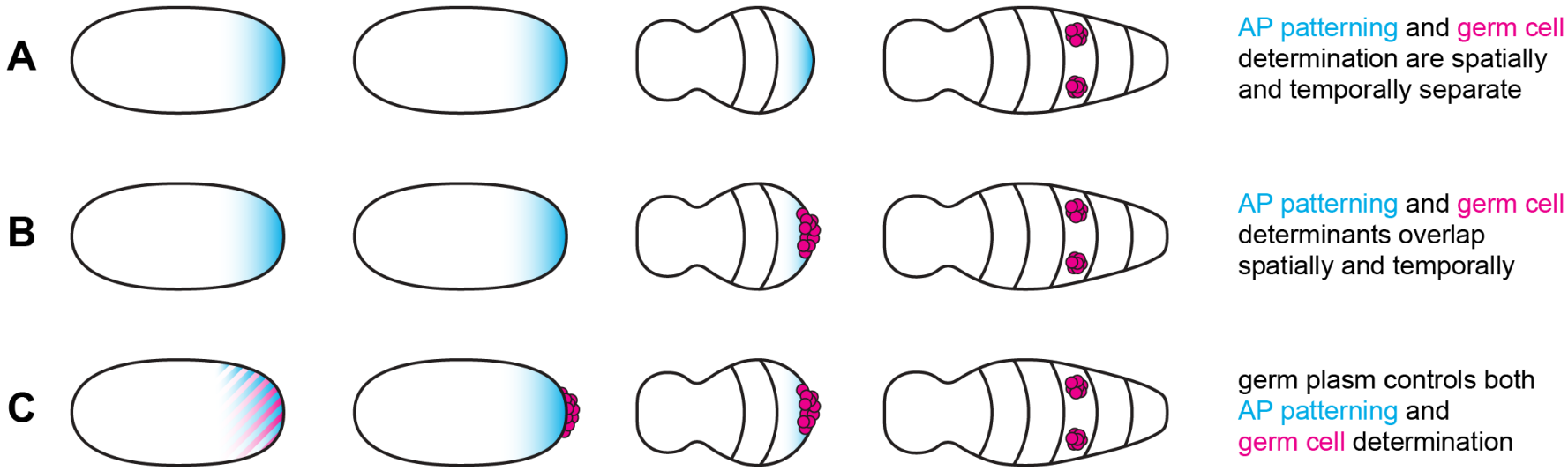


Figure 4. Hypothesized links between spatial and temporal dynamics of germ cell origination and the mechanism of germ cell specification. A) In species that induce germ cells later in embryogenesis (Category 3), the asymmetrically localized anterior–posterior (AP) patterning determinants (blue) and germ cell determination factors (pink) are spatially and temporally separate. B) The 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 couple germ cell specification and posterior patterning and thus promote the evolution of germ plasm.

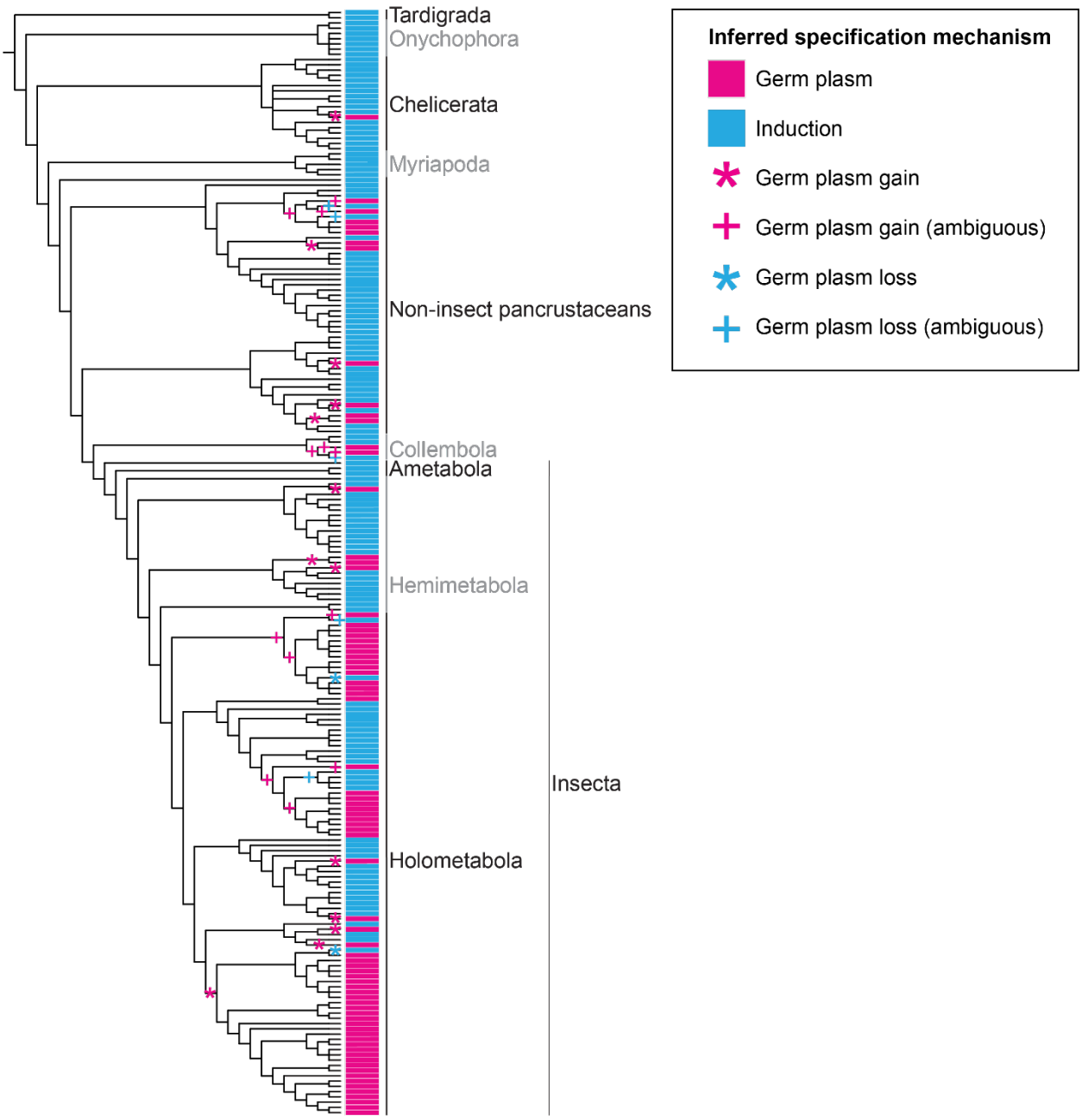


Figure 5. Evolutionary dynamics of panarthropod germ cell specification. Phylogenetic relationships among all study species are derived from published relationships described in the Supplementary Material, and branch lengths are not to scale. Boxes to the right of the cladogram designate the inferred mechanism of germ cell specification (pink = germ plasm, blue = induction). Pink asterisks denote instances of germ plasm evolution and pink pluses mark ambiguous cases of germ plasm gain. Blue asterisks denote instances of germ plasm loss and blue pluses mark ambiguous cases of germ plasm loss.

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