TITLE: Inferring a role for programmed cell death during the origin and evolution of wing polyphenism in ants

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ABSTRACT

Major evolutionary transitions in individuality, such as the evolution of multicellularity, occur when solitary individuals unite to form a single replicating organism with a division of labor between constituent individuals. Programmed Cell Death (PCD) has been proposed to play an important role during the origin and evolution of major transitions. Yet, it remains unclear to what extent PCD was involved in the major evolutionary transition to eusociality in ants, where solitary individuals united to form eusocial colonies with a division of labour between morphologically distinct queen and worker castes. Wing polyphenism is one of the principle morphological differences between queen and worker castes within a colony and is defined as the ability of an egg (same genome) to develop either into a queen with wings or into a worker that is completely wingless in response to environmental cues, such as nutrition and temperature. Because wing polyphenism evolved once at the origin of ants, it was likely intimately linked with the origin and evolution of eusociality. Here we test whether PCD plays a role in wing polyphenism in species from across the ant phylogeny encompassing taxa with both ancestral-like and derived characteristics. We show that PCD, mediated by the apoptosis pathway, is present in the rudimentary wing discs of worker larvae in 15 out of the 16 species tested. Using ancestral state reconstruction, we infer the presence of PCD in the last common ancestor of the poneroid and formicoid clades as well as all extant ants. Based on these results, our analysis predicts a role for PCD in regulating wing polyphenism during the evolutionary origin of ants. Implications for the role PCD in major evolutionary transitions to individuality are discussed.

INTRODUCTION

Major evolutionary transitions in individuality occur when solitary individuals unite to form a single replicating individual. During the history of life, such transitions include the evolution of eukaryotic cells from the integration of solitary prokaryotic cells, the integration of single-celled organisms to form multicellular organisms, the integration of solitary organisms to form eusocial colonies, and the integration of distantly-related organisms to form obligate endosymbioses (Buss, 2014; Maynard-Smith & Szathmary, 1997; West et al., 2015). Despite great advances made in understanding the evolutionary principles underlying these major transitions, the developmental mechanisms that facilitated these events remain poorly explored.

Recent advances in understanding the molecular and developmental processes during major transitions to multicellularity, has led to the proposal that programmed cell death (PCD) is a mechanism that plays an essential and general role in the emergence of major evolutionary transitions (Durand et al., 2019; Huettenbrenner et al., 2003). Unlike other modes of cell death, PCD is a genetically regulated process that occurs under specific physiological conditions (Galluzzi et al., 2018). PCD has ancient origins and is frequently found in unicellular eukaryotes and prokaryotes in addition to multicellular life (Ameisen, 2002). PCD is thought to play a role in facilitating major transitions by enhancing cooperation, formation of a morphological division of labor, and mediation of communication and conflict (Durand et al., 2019; Huettenbrenner et al., 2003; Libby & Ratcliff, 2014). For example, in some unicellular clonal or aggregate colonies, some individuals undergo PCD under stress conditions to provide nutrition to other members of the clonal group hence enhancing the survival and growth of the clones and thereby promoting group living – a precursor to multicellularity (Durand et al., 2014; Durand et al., 2011; Orellana et al., 2013). Moreover, experimental studies inducing multicellularity in yeast showed that the multicellular clusters fracture (via some cells undergoing PCD) allowing them to propagate into daughter cells (Ratcliff et al., 2012). Finally, in honeybees, PCD functions to degenerate ovaries in worker-destined larvae but not in queen-destined larvae (Durand et al., 2019; Hartfelder & Steinbruck, 1997). This led Durand et al. (2019) to propose that PCD may have played a role in the origin of reproductive division of labor in bees, and therefore, in a major transition to eusociality.

Here we test whether or not PCD contributed to the major evolutionary transition to eusociality in ants (family Formicidae) – a group that evolved eusociality only once (Boudinot, Richter, et al., 2022; Holldobler & Wilson, 1990). Concomitant with the evolution of eusociality, ants evolved a wing polyphenism, in which an egg can develop either into a winged queen or wingless worker in response to environmental cues, such as nutrition and temperature, that in turn influence hormone levels such as juvenile hormone (Hanna & Abouheif, 2021; Penick et al., 2012; Wheeler, 1986) (Fig. 1a). Wing polyphenism is a universal trait in ants (excepting for secondary losses) that evolved once at the origin of the group (Boudinot, Khouri, et al., 2022; Boudinot, Richter, et al., 2022; Hanna & Abouheif, 2021; Peeters, 2012; Perrichot et al., 2008). Ecological factors might have led to selection for a wing polyphenism in ants, where the wingless worker caste engaged in ground foraging and nesting while the winged reproductive

caste participated in nuptial flights. In the early lineages of ants, queens and workers are thought to have similar reproductive capacities, and the reproductive division of labor primarily occurs through behavioral regulation (Peeters, 1991). Therefore, the early evolution of wingless workers may have played an important role in the evolution of eusociality by limiting worker's participation in nuptial flight and dispersal, which in turn limited the ability of workers to mate and reproduce while also facilitating cooperation in brood rearing and foraging. Here, we ask if PCD contributed to a major evolutionary transition in eusociality by playing a key role in wing polyphenism in ants by degenerating the wings in the worker caste and limiting worker dispersal.

Individuals in the worker caste of ants, although completely wingless as adults, transiently develop wing discs during larval development (Abouheif & Wray, 2002; Dewitz, 1878; Rajakumar et al., 2018; Wheeler & Nijhout, 1981) (Fig. 1b–d, blue arrowheads). These wing discs are therefore considered as rudimentary organs, which Hall (2003) defines as 'an embryonic primordium of a more fully developed feature (the insect wing) found in an ancestor (a winged solitary wasp)'. Examining the highly conserved wing GRN in queen wing discs and worker rudimentary wing discs in derived ant species has revealed that the wing GRN is conserved between winged reproductive ants and other winged insects, but is interrupted in the wingless worker castes of different ant species (Abouheif & Wray, 2002; Shbailat & Abouheif, 2013; Shbailat et al., 2010). However, in two Amblyoponine species from the genus *Mystrium*, which are thought to reflect the ancestral characteristics of ants during the origin of eusociality, no interruptions were detected (Behague et al., 2018). This finding led Behague et al (2018) to propose that apoptosis (the most common genetic pathway for PCD) was co-opted to eliminate wing discs in the wingless worker caste during the origin of eusociality in ants. Although apoptosis has been previously detected in the worker rudimentary wing discs of two ant species (Sameshima et al., 2004; Shbailat et al., 2010), its presence across the ants and at the origin of wing polyphenism has yet to be tested.

The main goal of our study is to test for the role of PCD in wing polyphenism across the ant phylogeny. We do this by focusing on the two major clades of ants: the 'poneroid' and 'formicoid' clades. Species in the poneroid clade, like in the genus *Mystrium*, are of key importance to understanding the mechanism that may have been present in the early history of wing polyphenism, since these species exhibit several characteristics that are generally considered ancestral within ants (Burchill & Moreau, 2016; Keller & Peeters, 2022; Peeters, 1997; Wilson & Holldobler, 2005). Such characteristics are low queen-worker dimorphism, small colony size, and predatory behavior (Keller & Peeters, 2022; Wilson & Holldobler, 2005). In contrast, species in the formicoid clade largely exhibit derived social characteristics, such as high queen-worker dimorphism and subcaste diversification (Holldobler & Wilson, 1990; Wilson, 1953) We first test for the presence of apoptosis markers across a series of developmental stages in *Harpegnathos saltator*, a focal species with ancestral characteristics belonging to the poneroid clade. Second, we test for the presence of apoptosis markers in early and late stage larvae in an additional 15 species across the ant phylogeny, belonging to both the poneroid and formicoid clades. Finally, we conduct an ancestral state reconstruction analysis to infer whether or not PCD in wing polyphenism is present at ancestral nodes and at

154 the base of all extant ants. Studying the mechanism(s) operating during the origin of wing
155 polyphenism, and therefore, during or near the evolution of eusociality, will provide us with
156 insight on molecular and developmental mechanisms contributing to the emergence of a major
157 transitions in individuality during evolutionary history.
158
159 **RESULTS**
160
161 In *H. saltator*, we first tested for the presence of PCD in the rudimentary wing discs of worker-
162 destined larvae using the TUNEL assay, a marker of apoptotic cell death that detects DNA
163 fragmentation (Kyrylkova et al., 2012). We focused on the last larval instar (terminal stage)
164 beginning from the early-mid to the larval-pupal transition (Fig. 2). We found small amounts of
165 DNA degradation at the early and mid-periods in both the rudimentary wing pouch and hinge
166 (Fig. 2a', b' white arrowheads; Fig. 1b). Subsequently, during the terminal period, the amount of
167 DNA degradation increases substantially (Fig. 2c', d') and by the larval-pupal transition, TUNEL
168 signal is present at high levels throughout the entire rudimentary wing disc (Fig. 2e'). We then
169 tested for the presence of PCD in the wing discs of male-destined larvae, which will develop
170 into adult males with fully functional wings. We found that during the early and mid-period, the
171 amount and intensity of the TUNEL signal in the wing discs in male-destined larvae is much
172 lower as compared to that in the rudimentary wing discs of worker-destined larvae at similar
173 stages (Fig. 2f'–i' white arrowheads, compare with Fig. 2a'–d'). Moreover, during the larval-
174 pupal transition stage, where TUNEL expression is at its highest in the worker rudimentary wing
175 discs (Fig. 2e'), we find no signs of DNA degradation in the male wing discs (Fig. 2j'). Together,
176 these results suggest that the strong levels of DNA degradation in the worker rudimentary wing
177 discs relative to male wing discs is responsible for degrading the left-over rudiment after
178 growth has ceased, leading to the emergence of wingless worker adults. In contrast, the
179 presence of some DNA degradation in the male wing discs suggest that it may play a role in
180 shaping the final adult wings in males.
181
182 We next tested for PCD (TUNEL) in the rudimentary wing discs of worker-destined larvae across
183 15 additional species from five major subfamilies of ants (Fig. 3; Supp. 1). We then conducted
184 an ancestral state estimation analysis using data from all species tested to infer whether PCD
185 was present or absent in the rudimentary wing discs of workers in the last common ancestor of
186 ants. Like in *H. saltator*, we found positive TUNEL signal in the rudimentary wing discs during
187 late larval development in two other species belonging to the poneroid clade: *Odontomachus*
188 *brunneus* (Ponerinae) and *Stigmatomma pallipes* (Amblyoponinae) (Fig. 3a–c'). Moreover, we
189 observed a strong positive TUNEL signal in late-stage rudimentary worker wing discs in 12 out
190 of the 13 species we sampled from the formicoid clade (Fig. 3; Supp. 1). In 12 formicoid species,
191 TUNEL signal is predominantly present at high levels during the larval-pupal transition stage
192 (Fig. 3e', f', g', h', i', j', k'; Supp. 1), and in four species, it is also detected as early as the mid
193 period during last larval stage (Fig. 3f, h, i, j, arrowheads). *Tetramorium immigrans*
194 (Myrmicinae), which possesses very small rudimentary wing discs, is the only species where we
195 do not observe TUNEL signal at any stage examined (Fig. 3k–k'). Our ancestral state estimation
196 (under the equal rates model)) using data from all species tested strongly supports the presence
197 of PCD (>95% confidence) as being ancestral to all living ants, including the poneroformicine

198 clade (= poneroids + formicoids) (Supp. 2). Our inference remains robust after accounting for:
199 (1) uncertainty in the presence or absence of PCD in species from missing ant subfamilies; (2)
200 the absence of data for *Martialis* and Leptanillinae (the sister group to all other Formicidae);
201 and (3) different taxon sampling regimes (Supp. 2). Altogether, our experimental data and
202 ancestral state estimation analyses infer role for PCD in the regulation of wing polyphenism in
203 the last common ancestor of all extant ants.
204
205 Finally, we tested which specific forms of PCD are active in rudimentary wing discs in workers of
206 three ant species that belong to three different subfamilies. Although the TUNEL assay is a
207 general marker for apoptotic cell death, we sought to confirm the presence of apoptosis by
208 testing for the presence of the active form of caspase 3, the human ortholog of the *Drosophila*
209 ICE (Drice) protein. (Xu et al., 2009). We found strong cleaved-caspase 3 expression in the
210 rudimentary wing discs in workers of: *H. saltator* (Ponerinae), *Camponotus floridanus*
211 (Formicinae), and *Pheidole dentata* (Myrmicinae). In worker-destined larvae of *H. saltator* and
212 *C. floridanus*, cleaved-caspase 3 expression in the rudimentary wing disc starts around mid-
213 terminal larval development and spreads throughout the remaining larval stage (Fig. 4a–a', c–
214 c'). In *P. dentata*, we observe cleaved-caspase 3 expression only towards the late stages of
215 terminal development (Fig. 4d–d'). To test for the presence of autophagy, a type of PCD
216 characterized by the presence of vesicles (Yan et al., 2020), we used two different markers: atg8
217 and LC3A/B. The rudimentary wing discs of *H. saltator* and *P. dentata* workers show no
218 detectable signs of autophagy (Fig.4 b–b', e–e'). These experiments confirm that PCD in the
219 rudimentary wing discs of workers across ants is carried out via the apoptosis pathway.
220
221 **DISCUSSION**
222
223 PCD is a process that has been repeatedly co-opted to play a role in the regulation of
224 alternative phenotypes, such as polyphenism and sexual dimorphism, across the tree of Life
225 (Hanna & Abouheif, 2023). Here we show that PCD has again been co-opted to play a role in the
226 regulation of wing polyphenism in ants, a nearly universal trait in the group that evolved
227 concomitantly with eusociality in ants. We demonstrate that PCD through apoptosis, is strongly
228 expressed in late-stage rudimentary wing discs of workers in species sampled from across the
229 phylogeny of ants (Fig. 5 green circles). The presence of apoptosis in the rudimentary wing discs
230 of 15 out of 16 species we sampled across the poneroid and formicoid clades strongly suggests
231 that this process evolved once and is conserved across ants. Using formal ancestral state
232 estimation, we infer that PCD is ancestral to the poneroformicines (= poneroid + formicoid
233 clades) and the extant Formicidae as a whole (Fig. 6 green circles). This predicts PCD is present
234 and operating in the 'basal-most' subfamilies (Leptanillinae and Martialinae; see, e.g.,
235 Romiguier et al. (2022), for which wing polyphenism in females is also known to occur
236 (Borowiec et al., 2011; Chen et al., 2017; Hsu et al., 2017). In this context, rediscovery of
237 Martialinae will be of particular value, given that only two workers and a few males have been
238 collected to date (Boudinot, 2015; Rabeling et al., 2008). Furthermore, the occurrence of wing
239 polyphenism in females has been recently confirmed to be present among the extinct stem
240 lineages of ants (Boudinot, Khouri, et al., 2022), which supports PCD as a derived condition for
241 all Formicidae, extant and extinct (Fig. 5 dotted green circle). Future developmental studies

focusing on species in the Leptanillinae will be able to confirm the presence of PCD during the split of all extant ants.

PCD has contributed to the emergence of major evolutionary transitions in Earth's history by playing key roles in communication, conflict mediation, division of labor, and cooperation (Durand et al., 2019). Here we show that PCD very likely played a role in the origin of wing polyphenism in ants, and therefore, in the major evolutionary transition to eusociality in ant. By helping to generate a wingless worker caste, PCD may have been critical in reinforcing the reproductive division of labor in ancestral ant societies by limiting dispersal of workers, and therefore, their participation in mating flights. Multiple ecological, evolutionary, and developmental factors influenced the evolution of eusociality in ants and in other eusocial organisms. Here, we show that PCD may have been one of these factors during the origin of eusociality in ants.

Finally, many wasp taxa that are closely related to ants evolved a sexual wing dimorphism, where the females are wingless or short winged and the males are fully winged (e.g., XXX), or evolved wing polyphenism in females (*e.g.*, some Ichneumonidae, Cynipidae, Chalcidoidea, and Bethylidae). Given the overwhelming role of PCD in polyphenism and sexual dimorphisms across the tree of life (Hanna & Abouheif, 2023), we predict that it may be operating in wasp wing development to generate differences between the males and females or between female morphs within a species. This is a question of great interest because if PCD is present in these taxa, it would raise the intriguing possibility that wing polyphenism in ants may have originated from a latent potential for sexual wing dimorphism and polyphenism.

MATERIALS AND METHODS

Ant collection and colony care

Ant colonies were housed in plastic boxes lined with either fluon or talc powder. Artificial nests were constructed using glass test tubes half-filled with water and plugged with cotton. All colonies were maintained at 25 °C, 70% humidity and a 12h day:night cycle. Ants were fed a combination of mealworms, crickets, fruit flies, fruits, and Bhatkar-Whitcomb diet (Bhatkar & Whitcomb, 1970). *Harpegnathos saltator* colonies were housed in plaster nests and fed live crickets only 2-3 times per week.

Ant colonies were obtained from the following locals. *Stigmatomma pallipes* were collected at McGill Gault Nature Reserve (Quebec, Canada). *Lasius niger, Tetramorium immigrans, Aphaenogaster rudis*, and *Aphaenogaster picea* were collected at Mont Royal Park and McGill Gault Nature Reserve (Quebec, Canada). *Odontomachus brunneus* and *Camponotus floridanus* were collected at Gainesville (Florida, USA). *Pheidole dentata* were collected at Gainesville (Florida, USA) and Austin (Texas, USA). *Pheidole hyatti* were collected from Tempe (Arizona, USA). *Pheidole pallidula* were collected from Lyon (France). *Pheidole noda*, *Tetraponera rufonigra,* and *Polyrhachis rastellata* were collected from Mae Tang (Chiang Mai, Thailand). *Atta colombica* and *Acromyrmex echinatior* were collected from Gamboa (Panama). *Harpegnathos*

285 *saltator* colonies were propagated in the laboratory since the original collection in 1999 from
286 different locations in India.
287
288 **Immunohistochemistry, hybridization chain reaction, and TUNEL stainings**
289
290 **Fixation and Dissection**
291 Terminal stage larvae were collected and fixed as previously described by Shbailat and Abouheif
292 (2013). Fixed larvae were dissected using a Zeiss Discovery V12 stereomicroscope to expose the
293 wing discs and remove any obstructing fat tissue.
294
295 **Immunohistochemistry**
296 The following primary antibodies were used for immunohistochemistry: anti-cleaved caspase-3
297 (1:100-1:200, Cell Signaling Technology, #9661) and anti-LC3A/B (1:100, Cell Signaling
298 Technology, #4108). For all immunohistochemistry assays, fluorescent secondary anti-rabbit
299 polyclonal Alexa Fluor-555 (AbCam) antibody was used at 1:500 dilution to detect the primary
300 antibody, according to Khila and Abouheif (2008).
301
302 **Hybridization Chain Reaction**
303 To detect mRNA expression of *H. saltator atg8* we utilized the hybridization chain reaction
304 (HCR) methodology (Choi et al., 2018). Probe sets, amplifiers, and buffers were purchased from
305 Molecular Instruments, Inc. Procedure followed based on the Protocols for HCR^{TM} RNA-FISH
306 (v3.0) (generic sample in solution) acquired from molecularinstruments.com. Prior to
307 procedure, samples were fixed and dissected as described above, rehydrated in 25%, 50%, 75%
308 and 100% PTw (1X PBS; 0.1% Tween 20) and permeabilized in PTw and 2% Triton X-100 for 1
309 hour. Larvae were post-fixed in 4% formaldehyde in SSCT (5X sodium chloride sodium citrate;
310 0.1% Tween 20) for 2 hours and then washed 3 times with SSCT before moving into glycerol.
311
312 **TUNEL**
313 To detect programmed cell death in the wing discs of all species, we used the TUNEL (Terminal
314 deoxynucleotidyl transferase dUTP nick end labeling) assay. We used the *In Situ* Cell Death
315 Detection Kit, TMR red (Roche). Larval samples were fixed, dissected and stored in 100%
316 methanol in -30 °C prior to procedure. Dissected larvae were then rehydrated in 25%, 50%,
317 75%, and 100% PTw (1X PBS; 0.1% Tween 20). Samples were then washed 3 times (10 min
318 each) in freshly made PBT (1X PBS; 0.1% Triton; 0.1% BSA) and permeabilized in PTw and 2%
319 Triton X-100 for 1 hour, followed by 5 min washes in PBT. Positive and negative controls were
320 incubated in DNase buffer at 37°C for 10 minutes and subsequently washed with PBT for 5 min
321 (Supp. 3). Enzyme mix and label solution were added to the samples and the positive controls
322 (only label solution was added to negative controls) and incubated in the dark at 37 °C for 1
323 hour followed by 3 washes in PTw 5 min each. Samples were stained with DAPI (1:1000) for 1
324 hour to overnight and followed by gradual moving into glycerol. Samples were stored in final
325 concentration of 85% glycerol/DAPI before mounting and imaging.
326
327 **Imaging**

Fluorescent images of larval rudimentary wing discs of all species utilized in this study were taken using a Zeiss AxioImager Z1 microscope.

Ancestral state estimation

To reconstruct the ancestral conditions of PCD, we conducted a series of ancestral state estimation (ASEs) analyses given prior and new experimental data. To ensure that our estimates were based on well-supported phylogenetic results, we used the favored phylogram (P) and chronogram (C) of Romiguier et al. (2022), particularly as a recent simulation study has demonstrated the importance of branch lengths for ancestral state modeling (Wilson et al., 2022). We pruned the two trees (P, C) to three alternative taxon sets: (P1, C1) the first included only those terminals that match species with experimental data (17 taxa), (P2, C2) the second included experimental terminals plus *Martialis* and a leptanilline (22 taxa), and (P3, C3) the third included all ant terminals from the Romiguier et al. (2022) dataset in order to account for phylogenetic uncertainty (74 taxa). Because we had surplus experimental sampling of *Aphaenogaster* and *Pheidole* with identical results, we included only one species of the former and two species of the latter, as this matched the phylogenomic sampling of Romiguier et al. (2022). We also excluded non-ant outgroups as there is no applicable experimental data, thus insufficient information to parameterize a phylogenetic model that includes these taxa. As the 'all rates different' model overfit the data, resulting in complete statistical separation, we only report results under the 'equal rates' model.

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Figure 1

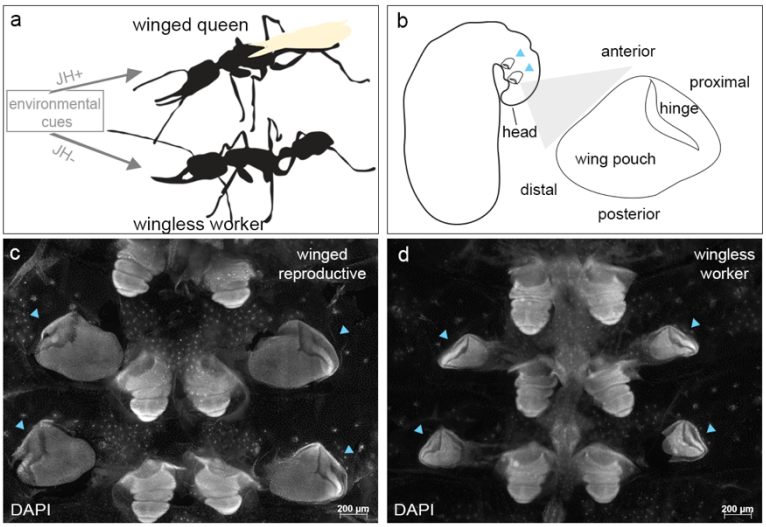


Figure 1. Wing polyphenism in the ponerine ant *H. saltator*: (a) environmental cues determine the development of winged queens (wings indicated in yellow) or wingless workers and reproductive workers 'gamergates'. (b) a cartoon illustration of *H. saltator* larva during terminal development indicating the location of the wing discs (blue arrowheads) and the general axes and regions of the wing disc in ants. Dissected terminal stage larvae in *H. saltator* stained with DAPI showing: (c) wing discs in winged males and (d) rudimentary wing discs in wingless female workers (blue arrowheads).

Figure 2

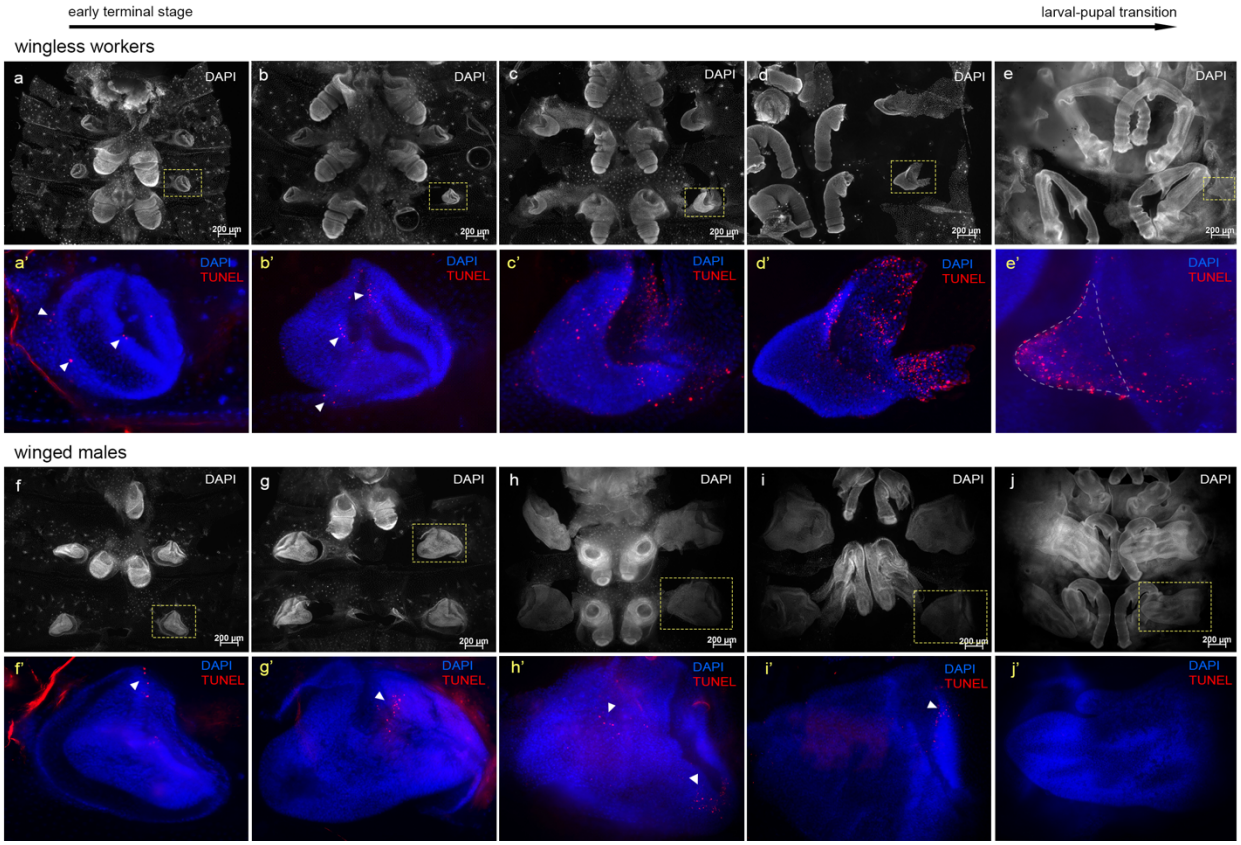


Figure 2. Programmed cell death in the wing discs of wingless workers and winged male larvae of *H. saltator*. (a-e) Larval developmental series in wingless female workers from early/mid terminal stage to larval-pupal transition stained with DAPI. (a'-e') a magnified view of a wing disc from the larva in the panel directly above showing TUNEL signal (red) and DAPI (blue). White arrowheads in (a', b') indicate area with TUNEL expression. (f-j) DAPI staining of a larval developmental series of winged males during terminal larval development corresponding in stage to those shown in the workers. (f'-j') a magnified view of a wing disc from the larva in the panel directly above showing TUNEL signal (red) and DAPI (blue). White arrowheads in (a', b' and f'-j') indicate region where TUNEL is expressed. Yellow dashed boxes in (a-e) and (f-j) indicate the wing disc magnified in the panels below. Images in panels (a-e) and (f-j) are to scale. Images in panels (a'- e') and (f'-j') are not to scale. All wing discs are oriented with distal axis on left and proximal axis on right (see Figure 1b).

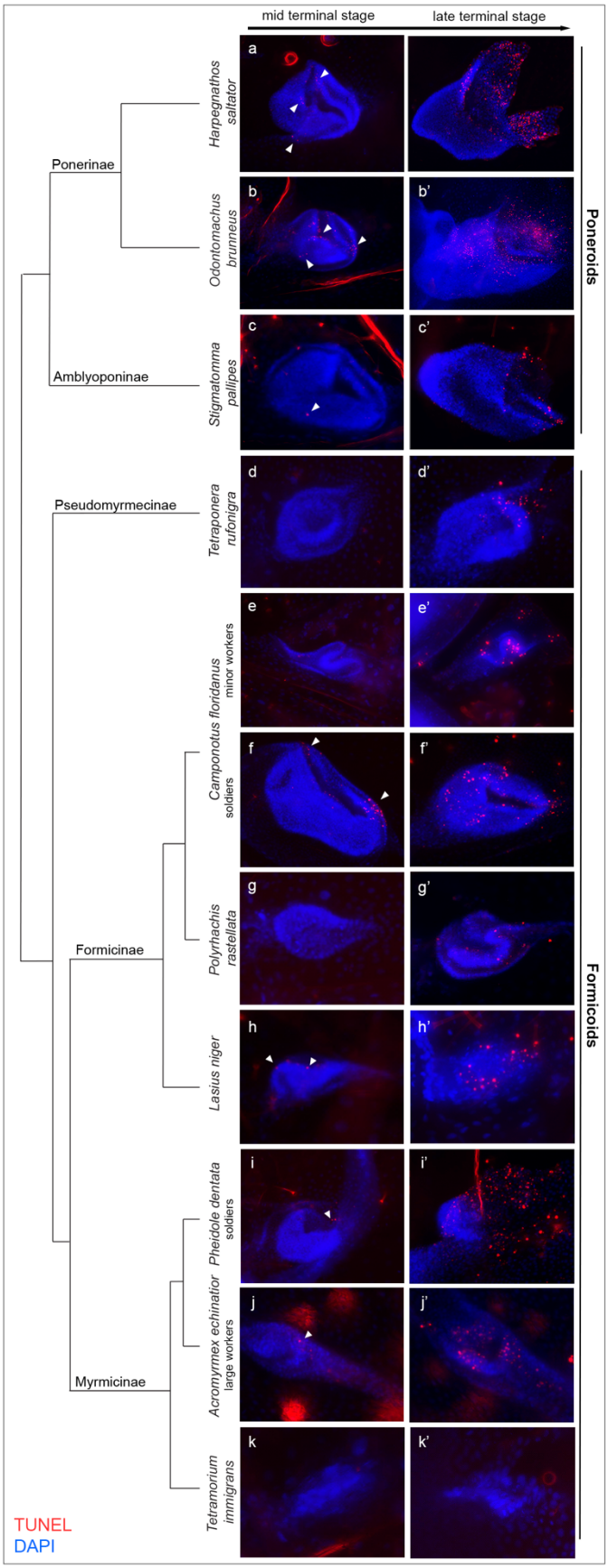
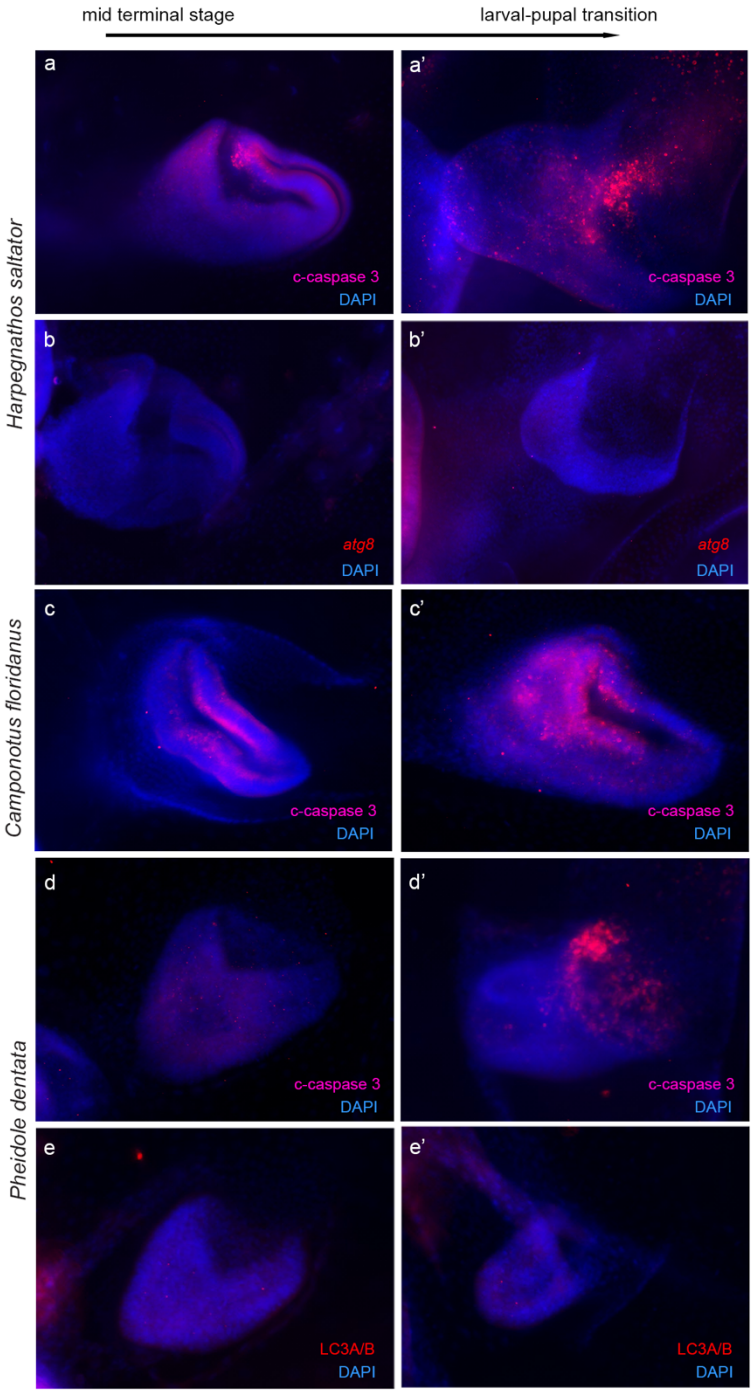


Figure 3

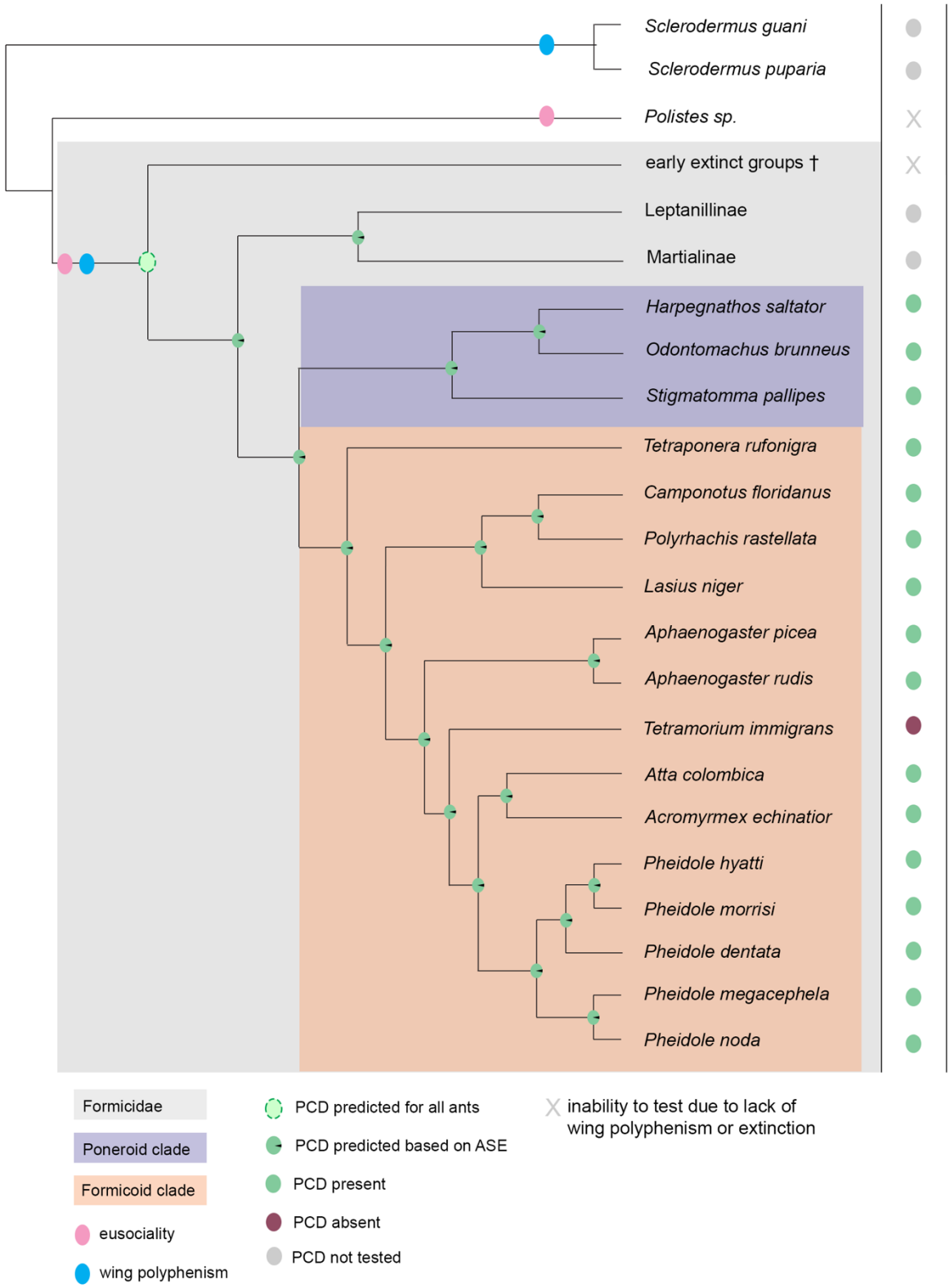
Figure 3. Programmed cell death in two stages of rudimentary wing disc development in larvae of wingless workers across the ant phylogeny. DAPI (blue) and TUNEL (red) signal in rudimentary wing discs of larvae in mid-terminal stage (left) and larval-prepupal transition stage (right). White arrowheads in panels (a, b, c, f, h, i, j) indicate region where TUNEL is expressed. Light black lines on the left mark phylogenetic relationships between the species represented. Subfamily labels are marked on the phylogenetic tree, species labels are marked on the left of the panels and clade labels are marked on the right-hand side of the panels. Images are not to scale. Phylogenetic relationship based on Borowiec et al (2019).

419 Figure 4
420



421
422 **Figure 4. Programmed cell death is carried out via the apoptosis pathway**. Expression of DAPI
423 (blue) and cleaved-caspase 3 (red) during mid (left) and late terminal larval (right) stages in *H.*
424 *saltator* (a-a'), and soldier larvae of *C. floridanus* (c-c') and *P. dentata* (d-d'). Expression of DAPI
425 (blue) and autophagy marker *atg8* or LC3A/B (red) in rudimentary wing discs of *H. saltator* (b-
426 b') and soldier larvae of *P. dentata* (e-e').
427

428 Figure 5
429



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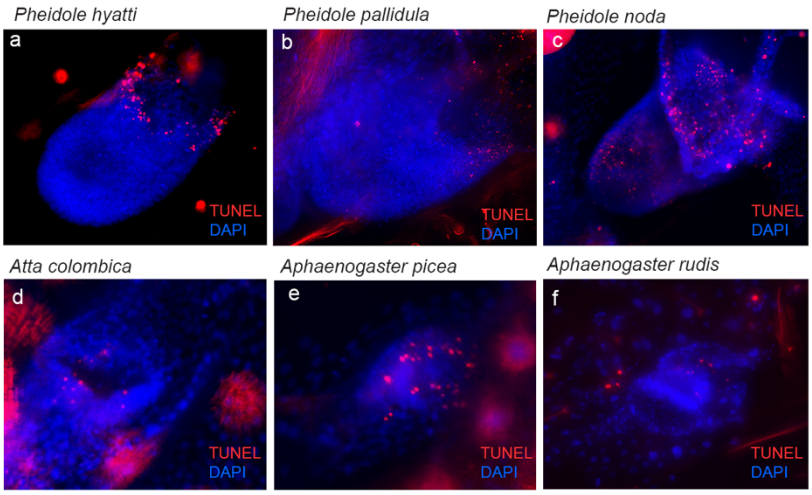
431

Figure 5. Programmed cell death in wing polyphenism across the ant phylogeny. Wing polyphenism (blue circle) and eusociality (pink circle) evolved concomitantly in ants (Formicidae, gray large box). Wing polyphenism and eusociality are found in some wasp species/groups. Outgroups for Formicidae are for demonstration purposes only and many branches in the Hymenoptera are excluded. Summary of PCD presence/absence found by this study for poneroid species (purple box) and formicoid species (orange box) are listed on the right. Characters indicate species with: presence of PCD (green circle), absence of PCD (red circle), PCD untested (gray circles). Gray X indicates species where testing is not possible. PCD regulation of wing polyphenism is predicted for all extant ants based on Ancestral State Estimations (ASE; green circles in nodes). ASE values for all tested models yielded over 95% confidence. PCD regulation of wing polyphenism at the origin of ants is proposed (dashed green circle). Branch length do not indicate evolutionary time. Phylogenetic relationships within the Formicidae are based on Boudinot, Khouri, et al. (2022); Moreau (2008); Ward et al. (2016).

446 Supplemental 1
447



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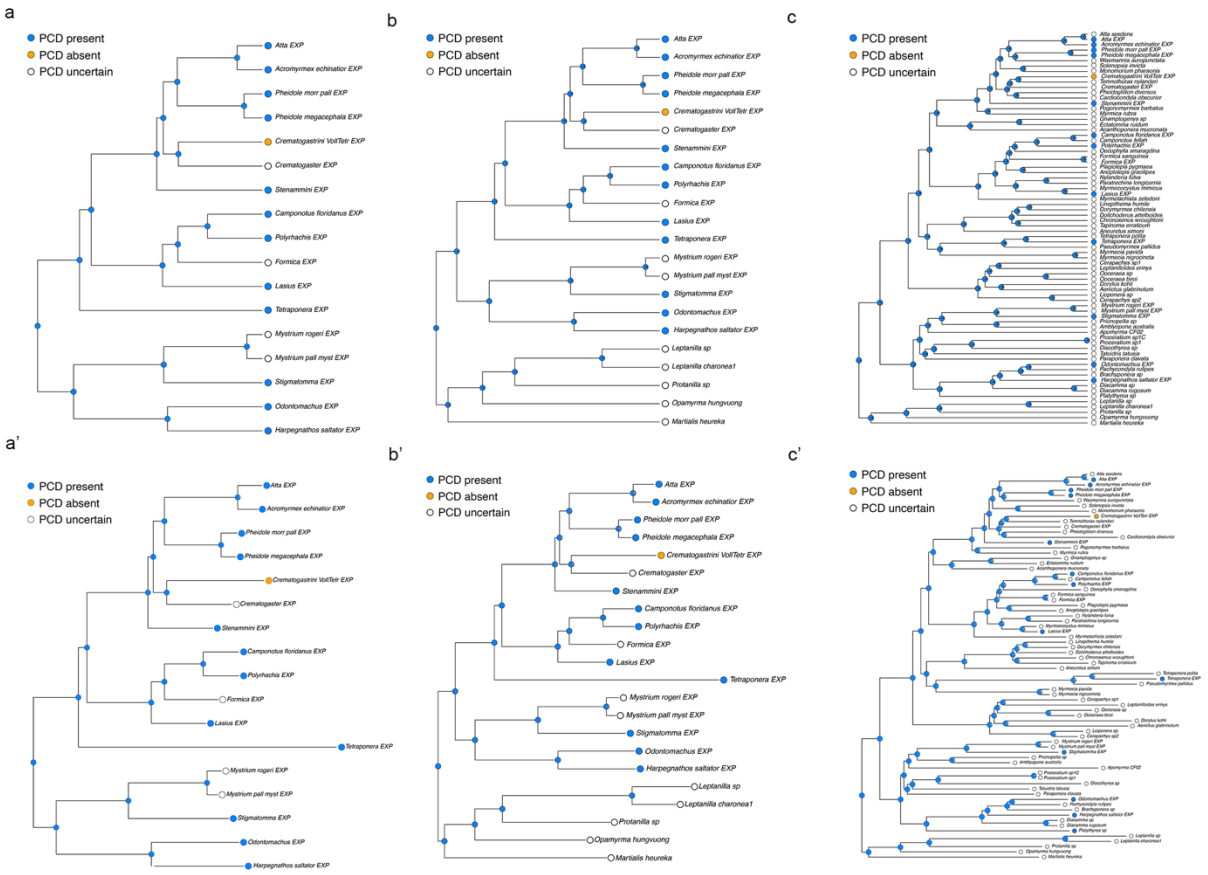
450 **Supplemental 1.** Programmed cell death in additional ant species from the subfamily
451 Myrmicinae. For all panels, a rudimentary wing discs at the late terminal larval stage is shown
452 stained in DAPI (blue) and TUNEL (red).
453

Supplemental 2



Supplemental 2. Ancestral State Estimations for presence and absence of PCD. Estimates based only on species where assays for PCD have been performed are shown using a phylogram (a) and chronogram (a’) modeling. Estimates based on species where assays for PCD have been performed as well as the addition of basal outgroups *Martialis* and leptanilline are shown using a phylogram (b) and chronogram (b’) modeling. Estimates based on 74 ant taxa including those without experimental data are shown using a phylogram (c) and chronogram (c’) modeling. Filled blue circles indicate estimates for presence of PCD; open circles indicate estimates for absence of PCD, open circle indicate species where PCD has not been examined. All models yielded over 95% confidence.

468 Supplemental 3

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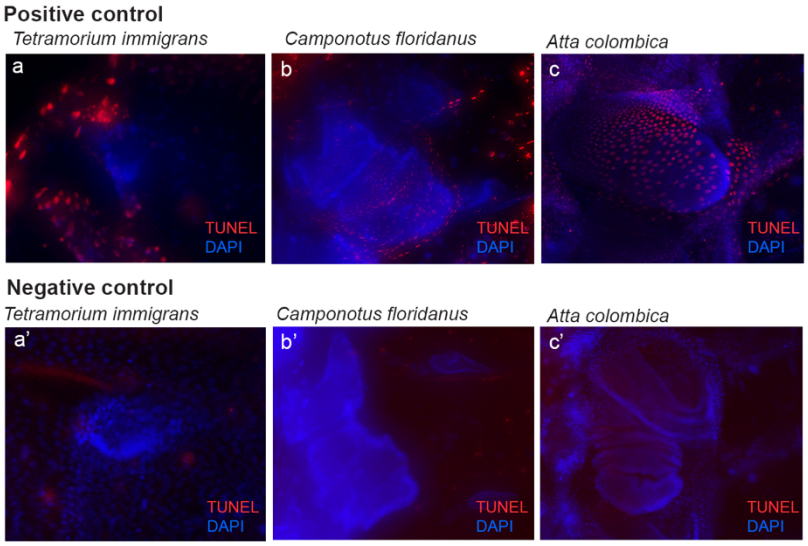

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472 **Supplemental 3.** Positive and negative TUNEL controls shown for three ant species. DAPI (blue),

473 TUNEL (red).

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