Thoracic crop formation is spatiotemporally coordinated with flight muscle histolysis during claustral colony foundation of a *Lasius japonicus* queen

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15 **ABSTRACT**

16 In a majority of ants, a newly mated queen independently founds a colony and claustrally raises her
 17 first brood without foraging outside the nest. During claustral independent colony foundation (ICF)
 18 in several ants, the esophagus of the founding queen expands and develops into a thoracic crop,
 19 which is then filled with a liquid substrate for larval feeding. It has been suggested that these
 20 substrates are converted from her body reserves (e.g., histolyzed flight muscles) or redistributed
 21 from a gastral crop. Here, we examined thoracic crop development in *Lasius japonicus* during
 22 claustral ICF. The foundresses claustrally fed their larvae from week 2 to 5 after ICF onset, and the
 23 first worker emerged at week 6. The development proceeded as follows: in week 0, foundress dorsal
 24 esophagus wall was pleated and thickened. Then, from week 2 to 5, the esophagus expanded dorsally
 25 toward where flight muscles had been present, following flight muscle histolysis. Gastral crop
 26 expansion followed esophagus expansion. Thus, thoracic crop formation may be spatiotemporally
 27 coordinated with flight muscle histolysis in *Lasius japonicus* queens, and similar developmental
 28 regulations might be common in other claustral ICF ants.

29

30

31 **Keywords;**

32 ant queen; claustral independent colony foundation; thoracic crop; flight muscle histolysis; *Lasius*

33

34

35 **INTRODUCTION**

36 Colonial foundation is a key step in the colony life cycle of eusocial hymenopterans. Colony

37 foundation strategies can be broadly divided into independent colony foundation (ICF), during
 38 which a foundress starts a colony alone, and dependent colony foundation (DCF), during which an
 39 existing colony is divided into two or more daughter groups (Cronin et al., 2013). Although DCF has
 40 evolved in more than 50 ant genera, ICF is a more widespread strategy and can be further divided
 41 into “non-claustral” and “claustral” ICF (Hölldobler and Wilson, 1990; Keller et al., 2014). In
 42 non-claustral ICF that is the most ancestral strategy in ants, a newly mated queen sheds her wings,
 43 excavates a new nest, and independently starts the colony (Peeters, 2020). During this founding
 44 period, the queen lays eggs, forages outside the colony, and raises her broods (Peeters and Molet,
 45 2010). On the other hand, during claustral ICF, which is derived from non-claustral ICF only in ants
 46 (Hölldobler and Wilson, 1990; Peeters, 2020; Wheeler and Buck, 1996), a founding queen also starts
 47 the colony independently but never forages outside the nest and raises her broods using only her
 48 body reserves (Brown and Bonhoeffer, 2003). Given that foraging ants experience a high mortality
 49 risk due to predation (Nonacs and Dill, 1990; Traniello, 1989), this claustral strategy allows ant
 50 queens to avoid foraging-associated risks (Brown and Bonhoeffer, 2003; Hölldobler and Wilson,
 51 1990). The acquisition of traits involved in claustral feeding has likely contributed to the ecological
 52 success of ants that employ such strategies. Indeed, the three subfamilies (Formicinae,
 53 Dolichoderinae, and Myrmicinae) that account for almost 80% of known ant species generally show
 54 claustral ICF (Peeters, 2020).

55 Claustral founding queens provide essential macronutrients such as lipids and proteins for their
 56 first larvae, and such macronutrients come from their body reserves. In claustral ICF species, young
 57 virgin queens possess enormous flight muscles (relative to their body mass) in the thorax and
 58 well-developed abdominal fat bodies (Hölldobler and Wilson, 1990; Peeters, 2020). Fat bodies are a

59 major source of lipids and storage proteins, e.g., hexamerin (Hahn et al., 2004; Wheeler and Buck,
60 1996, 1995). The lipids and proteins stored in the abdomens of founding queens are depleted during
61 claustral ICF, supporting the notion that these are transferred to the broods (Wheeler and Buck,
62 1996). In addition, voluminous thoraces with enormous flight muscles are a specialized traits for
63 claustral ICF (Peeters and Ito 2001). Because queens do not fly again after their mating flight, flight
64 muscles start to be histolyzed shortly after landing and release a large amount of protein resource
65 (Hölldobler and Wilson, 1990; Jensen and Børgesen, 2000; Wheeler and Buck, 1996). Flight muscle
66 histolysis of *Lasius niger* was originally described by Janet (1907) and then in greater detail by
67 Matte and Billen (2021).

68 In several ICF species, the founding queen's thoracic esophagus expands into a spindle shape
69 and is then called a "thoracic crop" (Janet, 1907; Jensen and Børgesen, 2000; Petersen-Braun and
70 Buschinger, 1975). This thoracic crop is thought to temporarily store the nutrients converted from
71 flight muscles and fat bodies as liquid food for larvae (Hölldobler and Wilson, 1990). Conversely,
72 Vander Meer et al. (1982) found that, in the fire ant *Solenopsis invicta*, the major component of the
73 thoracic crop (mainly triacylglycerols) originated in the gastral crop. Therefore, although thoracic
74 crop formation may serve as a key step in claustral feeding, this process is still poorly understood.

75 Here, we investigated the development of thoracic crop in the claustral ant *Lasius japonicus*
76 (Formicidae: Formicinae), which is one of the most common ants in Japan and closely related with
77 the congener *L. niger* (Maruyama et al., 2008). First, the timeline of claustral ICF, especially of
78 claustral feeding, were examined, and then, changes in sizes, morphologies, and internal structures
79 of the queen esophagus were examined during claustral ICF. Furthermore, it was investigated
80 whether such changes of esophagus were associated with either flight muscle histolysis or size

changes in gastral crop. Our results suggest that thoracic crop formation was spatiotemporally coordinated with flight muscle histolysis.

MATERIALS AND METHODS

Dealate queens of *Lasius japonicus* were collected from late June to mid-July in 2018, 2019, and 2021 at the Tamagawa University campus. Each founding queen with a body length of ca. 10 mm was kept in a plastic case (36 × 36 × 14 mm, AS ONE) with fully moistened plaster. Founding colonies were maintained at approximately 25°C during experiments, and queens were not fed.

During claustral ICF, the founding queen claustrally feeds her first larvae until the eclosion of the first worker. To better understand the claustral feeding timeline, a census was performed for ten experimental colonies and the number of eggs, larvae, pupae, and workers were counted weekly until six weeks after the queen's mating flight, when the first workers eclosed in the experimental colonies (see **RESULTS**). Because the founding queens in five of the ten experimental colonies died between weeks 5 and 6, the census at week 6 was performed only for the five remaining colonies.

To investigate the formation of thoracic crop, the queen esophagus morphology and size were examined at weeks 0–6 and also at week 8 when the first workers had eclosed in all colonies tracked. Ten to twelve founding queens were sampled from the other experimental colonies at weeks 0–6 and week 8 and then fixed in FAA fixative (formalin: acetic acid: ethanol = 6:1:16). The thoraces of the fixed queens were longitudinally dissected using a fresh razor blade and then photographed using a SZX10 stereo microscope (Olympus, Tokyo, Japan) equipped with a FLOYD digital camera (Wraymer, Osaka, Japan) and Wrayspect software (Wraymer). Because esophagus expansion

103 occurred in the mesothorax rather than in the prothorax and metathorax (see **RESULTS**), the
104 esophagus height in the mesothorax was measured as a proxy for esophagus size. Flight muscle
105 histolysis was examined simultaneously.

106 To investigate the formation of the thoracic crop in more detail, we used the Transparentizing
107 and Autofluorescence Scanning (TAFS) method, which enables the observation of the morphologies
108 of decolorized and transparentized samples without staining using the autofluorescence of the
109 samples themselves. Founding queens at weeks 0 and 3 were fixed with FAA and stored in 70%
110 EtOH. These samples were then decolorized in a 6% hydrogen peroxide/70% EtOH solution for at
111 least one week (Fig. S1A, B). Then, the samples were dehydrated in ascending series of isopropanol
112 and transparentized in a BABB solution (benzyl alcohol/benzyl benzoate = 1:2) for four days (Fig.
113 S1C). The *Lasius* queens were too large for whole-mount confocal microscopy, so they were sliced
114 into 500 µm thick pieces using LinearSlicer PRO7 (D.S.K., Kyoto, Japan) (Fig. S1D). Sliced
115 samples were examined with an Olympus FV3000 Confocal Laser Scanning Microscope (Olympus)
116 using excitation lasers at wavelengths of 405, 473, and 559 nm. Fluorescence excited by lasers with
117 different wavelengths was recorded in different channels and superimposed later. The acquired
118 confocal images were three-dimensionally constructed and analyzed by Imaris software ver. 7.0.0
119 (Bitplane AG, Belfast, United Kingdom). Since autofluorescence differs among tissues, and under
120 similar levels of excitation, the TAFS method visualizes differences in autofluorescence
121 characteristics across tissues as differences in “color.”

122 To examine whether the thoracic crop contents originated in the gastral crop, the gasters
123 (consisting of the posterior parts than third abdominal segments) of the FAA-fixed queens were
124 dissected, and the lateral areas of the dissected crops were measured as a proxy for gastral crop size.

125 All statistical analyses were conducted using R ver. 4.1.0 (available at <http://cran.r-project.org/>).

127

128

129 **RESULTS**

130

131 **Timeline of claustral larval feeding during *L. japonicus* colony foundation**

132 Following their mating flights, founding queens laid 35.2 ± 14.5 eggs (mean $\pm$ SD) in the first week
133 and then continued to lay approximately 40 eggs (38.8 ± 20.6) per week for six weeks (Fig. 1 and
134 Fig. S2). Larvae first emerged at week 2 and then peaked in abundance at week 3 (Fig. 1). In
135 addition, some larvae cannibalized an egg or another larva during the six weeks (Fig. S3). Pupation
136 and eclosion were first confirmed at weeks 4 and 6, respectively (Fig. 1). These results indicate that
137 founding queens claustrally feed their larvae at least from weeks 2 to 5, assuming that anatomical
138 changes associated with claustral feeding should occur during or ahead of this period.

139

140 **Thoracic crop formation parallel to flight muscle histolysis**

141 Flight muscle degeneration began in week 1, and then, almost all muscle fibers were empty by
142 week 3 (Fig. 2). The esophagus gradually expanded during ICF in parallel with flight muscle
143 histolysis (Fig. 2). The esophagus after week 2 appeared larger than it did during weeks 0 and 1
144 (Fig. 2). Esophagus height increased significantly over time (one-way ANOVA, $F = 9.287$, $P <$
145 0.001), and at its peak height (week 5) was four times greater than its height at week 0 (Fig. 3).
146 When the expanded esophagus was accidentally broken at weeks 3 and 5, it exuded a yellowish,

147 oily substrate.

148 The TAFS analyses revealed that the internal structure and size of the queen's esophagi had
149 changed during claustral ICF. At week 0, the thoracic esophagus was not yet distended as
150 mentioned above (Fig. 4A), and the flight muscles had not yet decomposed, so muscle fiber
151 structures were still recognizable (Fig. 4B). The esophagus wall was thicker and pleated
152 throughout the thoracic esophagus (Fig. 4C), and two layers with different autofluorescence
153 characteristics were observed (Fig. 4D–H). Of these two layers, the outer layer (topologically
154 inside of the body) was almost uniform in thickness throughout the thoracic esophagus, whereas
155 the thickness of the inner layer (topologically outside the body) was spatially varied. The inner
156 layer was more thickened on the dorsal side (Fig. 4G) than on the ventral one (Fig. 4H) in the
157 prothorax, mesothorax, and metathorax (Fig. 4D–F). In addition, the inner layer of the dorsal
158 wall had thickened further in the mesothorax (Fig. 4E, G, and Fig. S4D) than it had in the
159 prothorax and metathorax (Fig. 4D, F). In contrast, the esophagus wall on both the ventral and
160 dorsal sides, where such bilayer structures could not be recognized, was thinner and less pleated
161 in the petiole (Fig. 4I–K).

162 At week 3, the thoracic esophagus swelled to a spindle shape (Fig. 5A), and the flight
163 muscles were degraded enough that no fiber structure was recognizable (Fig. 5B). The
164 esophagus wall was thinly stretched and expanded to the dorsal side, particularly in the
165 mesothorax (Fig. 5C and Fig. S4E), and wall thickness was almost uniform throughout the
166 thoracic esophagus (Fig. 5D–F). The surface of the expanded esophagus showed rumpled
167 structures extending parallel to the anterior–posterior axis (Fig. 5C), and the two-layered inner
168 structures observed at week 0 had become unrecognizable (Fig. 5G). These results indicate that

169 the founding queens form thoracic crops by week 2 by expanding their esophageal inner wall,
170 particularly where it was thickened and pleated at week 0.

171

172 **Changes in gastral crop size**

173 Gasteral crop size did not change for four weeks after the mating flight, but it did become
174 significantly larger thereafter (one-way ANOVA, $F = 8.448$, $P < 0.001$, Fig. 6), indicating that crop
175 size changed following esophagus expansion.

176

177

178 **DISCUSSION**

179 During claustral ICF in *Lasius japonicus*, a founding queen's flight muscles were histolyzed in the
180 first three weeks as shown in *L. niger* (Matte and Billen, 2021), and esophageal expansion began in
181 week 2 in parallel to the initiation of larval demand for nutrition (Fig. 7). The expansion was more
182 evident in the esophagus's dorsal wall than in the ventral wall, and the expanded esophagus occupied
183 a space in the dorsal side of the thoracic cavity where flight muscle histolysis had occurred. Thus,
184 our study shows that thoracic crop formation is spatiotemporally coordinated with flight muscle
185 histolysis in the claustral founding queen of *L. japonicus*. In addition, the inner layer of the dorsal
186 esophagus wall at week 0 was thicker and more pleated than that of the ventral wall, in the
187 mesothorax where the thoracic crop was formed after week 2. The rapid expansion of the esophagus
188 within the first three weeks of claustral ICF may be facilitated by stretching these thickened and
189 pleated esophagus walls. The expansion of the dorsal esophagus wall in the claustral founding queen
190 has been reported in five species from two subfamilies (Myrmicinae and Formicinae) (Jensen and

191 Børgesen, 2000; Petersen-Braun and Buschinger, 1975). In addition, pleats in the dorsal esophagus
192 wall were reported in newly eclosed queens of the Myrmicinae ant *Monomorium pharaonis*
193 (Petersen-Braun and Buschinger, 1975). Therefore, the pleating, thickening, and expansion of the
194 dorsal esophagus wall are likely adaptations that enable thoracic crop formation in spatiotemporal
195 coordination with flight muscle histolysis, which would be a common process in claustral ant
196 species.

197 As reported in other ants, the thoracic crop of *L. japonicus* was filled with yellowish and oily
198 substrates during the claustral feeding period (Petersen-Braun and Buschinger, 1975), which would
199 provide nutrition for larvae. Thoracic crop formation occurred prior to gastral crop expansion (Fig.
200 7). In contrast to the previous study showing that lipids (mainly triacylglycerols) stored in a gastral
201 crop flowed into the thoracic crop in fire ant queens (Vander Meer et al., 1982), nutritive resources
202 within the thoracic crop of *L. japonicus* queens do not derive from, but would rather flow into, the
203 gastral crop. As Hölldobler and Wilson (1990) suggested, the liquid stored in the thoracic crop might
204 derive from converted queen tissues, such as metabolized fat bodies and flight muscles. Unmated,
205 young queens of claustral species generally contain a far higher relative fat content than those of
206 non-claustral species (Keller and Passera, 1989). Moreover, their fat content is depleted during
207 claustral founding but not during mating flight (Passera et al., 1990; Tschinkel, 1993), suggesting
208 that the fat reserves were utilized for the queen's own survival, egg laying, and larval feeding during
209 claustral ICF. However, in *Camponotus japonicus*, the founding queen would provide the first larvae
210 with protein reserves rather than fat reserves (Idogawa et al., 2017). In addition, in queens of
211 *Crematogaster opuntiae* (Myrmicinae) and *Camponotus festinatus*, protein reserves in both thoraces
212 and gasters were also depleted during claustral ICF (Wheeler and Buck, 1996). The flight muscles

213 were much enlarged in queens of claustral species than in those of non-claustral species (Keller et al.,
214 2014), and thus, their thoraces with enlarged flight muscles were suggested to be a hallmark of
215 specializations for claustral ICF (Peeters and Ito, 2015, 2001). The coordination between thoracic
216 crop formation and flight muscle histolysis indicates that histolyzed flight muscles could provide a
217 major source of thoracic crop content in *L. japonicus*. Further investigations are needed to reveal
218 how such metabolized reserves are converted and stored in the thoracic crop.

219 We also found that during claustral ICF, some larvae cannibalized a sister egg (we could not
220 discriminate between a reproductive or trophic egg) or larva. The ant larvae were suggested to be
221 generally forced into a cannibalistic diet, especially oophagy, during claustral ICF (Urbani, 1991).
222 Furthermore, the claustral queen of *S. invicta* and *Atta sexdens* (Myrmicinae) ingested trophic eggs,
223 and then, the *Solenopsis* queen regurgitated it to larvae (Augustin et al., 2011; Cassill, 2002). Thus,
224 the claustral queens would also convert a portion of their own metabolized body reserves into eggs,
225 and then they may provide a portion of their eggs (or larvae) to existing larvae directly (Hölldobler
226 and Wilson, 1990) or may regurgitate eggs ingested by themselves (Cassill, 2002; Meurville and
227 LeBoeuf, 2021).

228 Our study described the process of thoracic crop formation in *L. japonicus* queens, which would
229 be regulated to be spatiotemporally coordinated with flight muscle histolysis. Such developmental
230 regulations for thoracic crop formation in a founding queen might be ubiquitous in claustral ICF
231 species. In non-claustral ICF species, flight muscle histolysis occurs in founding queens (Haskins,
232 1941; Matte and Billen, 2021), but whether queen esophagus morphology changes during
233 non-claustral ICF has not yet been examined. Conversely, thoracic crops have been reported in
234 workers of various species, including non-claustral ponerine species (reviewed in Casadei-Ferreira et

al., 2020). Therefore, developmental regulations for thoracic crop formation acquired in workers of more primitive ant lineages might have been co-opted in the founding queens of claustral ICF species. Future studies comparing founding queen internal structures, particularly those on the esophagus, during ICF between claustral and non-claustral species will enable us to examine the evolutionary process of thoracic crop in the claustral ICF ant queens.

AUTHOR CONTRIBUTION

Kurihara: Investigation, Formal analysis, Visualization, Writing – original draft; **Ogawa:** Methodology, Investigation, Visualization, Writing – review & Editing; **Chiba:** Investigation, Writing – Review & Editing; **Hayashi:** Conceptualization, Funding acquisition, Writing – review & Editing; **Miyazaki:** Conceptualization, Funding acquisition, Project administration, Writing – review & Editing, Supervision.

ACKNOWLEDGMENTS

We thank Hiromi Tanaka, Toshimi Hidaka, and other lab members in Tamagawa University for their assistance in field sampling, colony maintenance, and laboratory work. This work was supported by JSPS KAKENHI Grant Numbers JP19K06860 to SM and JP20K06816 to YH. The authors would like to thank Enago (www.enago.jp) for the English language review.

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318

319

320 **FIGURE LEGENDS**

321 **Figure 1.** Mean offspring number produced by *Lasius japonicus* founding queens during claustral

322 ICF. Individual plots for each developmental stage (egg, larva, pupa, and adult worker) are shown in

323 Figure S2. Week 0 corresponds to the day of their nuptial flight.

324

325 **Figure 2.** Queen esophagus and flight muscles during claustral ICF. “w0–w6” at the bottom left of
326 each panel indicates the claustral ICF stage, i.e., 0–6 weeks after mating flight. *fm* and arrowhead
327 indicates flight muscles and esophagus, respectively. Scale bars 500 μ m.

328

329 **Figure 3.** Changes in the esophagus height of a queen during claustral ICF. Each box-and-whisker
330 plot indicates the median (horizontal line), interquartile range (box), and distance from the upper and
331 lower quartiles times 1.5 interquartile range (whisker). All individual data shown as gray plots.
332 Different letters indicate significant statistical differences ($P < 0.05$, Tukey–Kramer tests for
333 post-hoc multiple comparisons).

334

335 **Figure 4.** TAFS images of the thorax and petiole of a queen at week 0 in claustral ICF. Parasagittal
336 sections of the (A) thorax and petiole, (B) flight muscle (dorsal longitudinal muscle), and (C)
337 esophagus. Parasagittal sections of the esophagus in the (D) prothorax, (E) mesothorax ([G] dorsal
338 and [H] ventral walls), (F) metathorax, and (I) petiole ([J] dorsal and [K] ventral walls). Thoracic
339 esophagus consists of two layers, namely, outer (dark blue) and inner layers (light blue). Letters with
340 single quotes indicate transverse sections marked by respective letters.

341

342 **Figure 5.** TAFS images of queen thorax and petiole at week 3 of claustral ICF. Parasagittal sections
343 of the (A) thorax and petiole, (B) degenerating flight muscle (dorsal longitudinal muscle), and (C)
344 expanding thoracic esophagus ([G] dorsal wall in the mesothorax). Transverse sections of the

345 esophagus in the (D) prothorax, (E) mesothorax, and (F) metathorax. Arrowheads indicate the
346 esophagus wall, depicted in blue.

347

348 **Figure 6.** Size changes in the gastral crop of a queen during claustral ICF. The areas of the gastral
349 crop photographed from the lateral view (inset, scale bars 300 μm) were measured as a gastral crop
350 size. Each box-and-whisker plot indicates the median (horizontal line), interquartile range (box), and
351 distance from the upper and lower quartiles times 1.5 interquartile range (whisker). All individual
352 data are shown as gray plots. Different letters indicate significant statistical differences ($P < 0.05$,
353 Tukey–Kramer tests for post-hoc multiple comparisons).

354

355 **Figure 7.** Summarized timeline of thoracic crop formation, flight muscle histolysis, and gastral crop
356 expansion, as well as changes on larval demands for feeding, during claustral ICF in *Lasius*
357 *japonicus*.

358

359

360 **Figure S1.** Sample preparation process for TAFS microscopy. (A) A FAA-fixed sample was (B)
361 decolorized, (C) transparentized, and then (D) sliced to 500 μm thicknesses.

362

363 **Figure S2.** Offspring number at each developmental stage (egg, larva, pupa, and adult worker)
364 produced by *Lasius japonicus* founding queens during claustral ICF.

365

366 **Figure S3.** Larvae cannibalizing (A) egg and (B) larva in the claustral founding colonies. Scale bars

367 500 μm .

368

369 **Figure S4.** Changes in the surface structures of the thoracic esophagus in a decolorized sample.

370 Dorsal view of the (A) queen thorax and dissected thoracic esophagus at (B, D) week 0 and (C, E)

371 week 5 in claustral ICF.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7