

Ergatoid queen development in the ant *Myrmecina nipponica*: modular and heterochronic regulation of caste differentiation

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Caste polyphenism in social insects provides us with excellent opportunities to examine the plasticity and robustness underlying developmental pathways. Several ant species have evolved unusual castes showing intermediate morphologies between alate queens and wingless workers. In some low-temperature habitats, the ant *Myrmecina nipponica* produces such intermediate reproductives (i.e. ergatoids), which can mate and store sperm but cannot fly. To gain insight into the developmental and evolutionary aspects associated with ergatoid production, we conducted morphological and histological examinations of the post-embryonic development of compound eyes, gonads and wings during the process of caste differentiation. In compound eyes, both the queen-worker and ergatoid-worker differences were already recognized at the third larval instar. In gonads, queen-worker differentiation began at the larval stage, and ergatoid-worker differentiation began between the prepupal and pupal stages. Wing development in ergatoids was generally similar to that in workers throughout post-embryonic development. Our results showed that the developmental rate and timing of differentiation in body parts differed among castes and among body parts. These differences suggest that the rearrangement of modular body parts by heterochronic developmental regulation is responsible for the origination of novel castes, which are considered to be adaptations to specific ecological niches.

Keywords: caste development; heterochrony; imaginal primordia; modularity; social insect

1. INTRODUCTION

Phenotypic plasticity refers to the emergence of adaptive phenotypic variations in a developing organism in response to varying environmental conditions. Phenotypic plasticity is thought to contribute to phenotypic evolution and speciation (Hall 1999; West-Eberhard 2003). One type of phenotypic plasticity is ‘polyphenism’, in which discrete alternative phenotypes are produced in response to extrinsic factors (Nijhout 1999, 2003). In polyphenisms, when the canalization of forked developmental pathways that occur at certain points in evolutionary time is not necessarily robust, the occasional production of intermediate phenotypes, or the addition of alternate pathways to the original polyphenic developmental routes, can result. Caste differentiation in social insects provides us with excellent opportunities to evaluate the robustness and flexibility of the alternative canalized developmental pathways observed in polyphenism (Nijhout 2003; Miura 2005). In caste differentiation, the multiple developmental pathways that produce discrete castes are considered to reflect the

canalized conditions. In nature, however, it is sometimes possible to observe intermediate castes in a number of social insect species (Peeters 1991; Heinze 1998; Miura 2005).

One of the major factors underlying the evolutionary success of social Hymenoptera, an ecologically dominant group, is the division of labour between reproductive and non-reproductive castes (Hölldobler & Wilson 1990; Ward 2006). Although genetic factors are invoked to affect caste determinations in many ant species (Winter & Buschinger 1986; Fraser *et al.* 2000; Volny & Gordon 2002; Helms *et al.* 2003; Hughes *et al.* 2003; Buschinger 2005; Smith *et al.* 2008), castes in most social hymenopterans are primarily determined by extrinsic factors such as nutrition (Wheeler 2002). When colony members that share similar, sometimes identical, genetic backgrounds receive different nutritional cues during larval stages, their developmental pathways are differentiated, resulting in caste-specific phenotypes (Wheeler 1986; Nijhout 1999; Miura 2005). Many ant species (Formicidae) produce at least two morphologically distinct female castes, i.e. queens and workers. Queens generally have a well-developed thorax with deciduous wings for nuptial flight and are able to reproduce and fly, whereas workers have a simplified thorax without wings and lose (or have reduced) reproductive and flight abilities.

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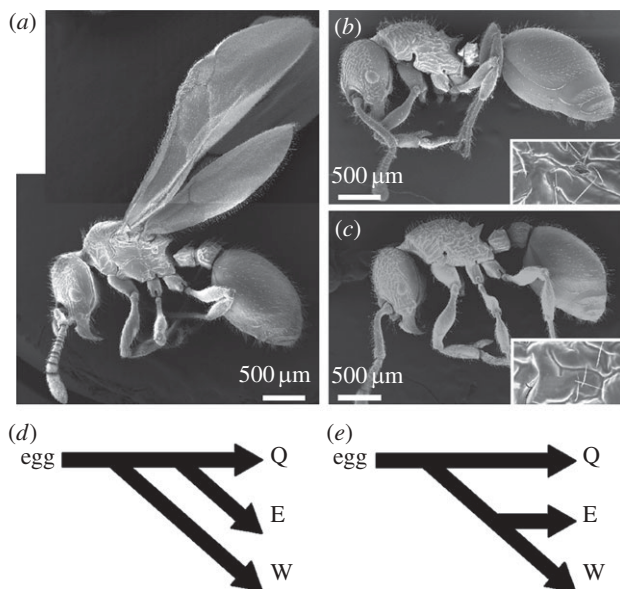


Figure 1. Photographs of three female castes: winged queen, ergatoid and worker, and two hypothetical caste differentiation pathways in *M. nipponica*. (a) Whereas the queen has functional wings and a well-developed thorax, (b) the ergatoid and (c) the worker are wingless and have simplified thoraxes. Some ergatoids have rudimentary wing buds, which appear as small holes or projections in the mesothorax (b, inset). The scale bars in the insets in (b,c) indicate 50 μm . Well-developed compound eyes and gasters comparable with queens are also ergatoid-specific characters (Miyazaki *et al.* 2005). As ergatoids are thought to arise from modified ancestral queen-worker differentiation pathways, two hypothetical pathways leading to the production of ergatoids are presented: (d) ergatoids derived from queen larvae (hypothesis I) and (e) from worker larvae (hypothesis II). Q, queen; E, ergatoid; W, worker.

However, as mentioned previously, certain ant species produce more than two female castes, such as multiple worker castes (Oster & Wilson 1978). These polymorphic worker castes, defined as subcastes, perform specific tasks. Some species also produce several types of reproductives: multiple types of alate queens with different wing sizes (Rüppel & Heinze 1999), alate/wingless reproductives (Winter & Buschinger 1986; Heinze & Buschinger 1987; Peeters & Hölldobler 1995; Fersch *et al.* 2000; Buschinger & Schreiber 2002), or a single type of wingless reproductive instead of alate queens (Peeters & Higashi 1989; Tsuji *et al.* 2001; Hölldobler *et al.* 2002; Molet *et al.* 2006). As these diversifications represent deviations from ancestral caste developmental patterns (Wheeler 1986; Peeters 1991), comparisons of derived developmental pathways with the ancestral pathways give us insight into the modifications of canalization patterns, which may have led to the phenotypic evolution (figure 1d,e).

In this study, we focused on *Myrmecina nipponica*, which generally has two female castes: an alate queen (figure 1a, hereafter referred to as a 'queen') and a worker (figure 1c). However, some colonies of this species possess wingless reproductives instead of queens (figure 1b; Ohkawara *et al.* 1993). This wingless reproductive caste has been reported as an 'intercaste' (Ohkawara *et al.* 1993) or an 'intermorphic queen' (Murakami *et al.* 2002), but we refer to this caste as an

'ergatoid queen' (hereafter referred to as an 'ergatoid'). Generally, wingless reproductive castes are either 'gamergates', in species where workers can mate, or 'ergatoids', when they are morphologically different from workers (Molet *et al.* 2009). *Myrmecina nipponica* is widespread in Japan, and colonies normally consist of queens and wingless workers in most populations; we refer to these colonies as 'alate-queen colonies'. Colonies consisting of workers and ergatoids, designated as 'ergatoid colonies', have been reported at two locations. These locations also include alate-queen colonies, although both types of reproductives rarely coexist in the same colony (Ohkawara *et al.* 1993). Experimentally, however, ergatoids can also be induced from alate-queen colonies by low-temperature treatments (Murakami *et al.* 2002). This result is consistent with the fact that the two locations are relatively colder than other areas inhabited by this species; one is in the northernmost margin of their range (Hokkaido) and the other is further south but still at a high altitude (e.g. at 1300 m elevation of Mt. Takanawa, Ehime, F. Ito 2009, personal communication), suggesting that the production of ergatoids may be triggered by low temperature.

In ergatoids, the development of body parts is not just intermediate in every body part (Miyazaki *et al.* 2005). Ergatoids have well-developed ovaries, spermathecae and compound eyes, similar to those of queens, but they have reduced thoracic parts, similar to those of workers (Ohkawara *et al.* 1993; Murakami *et al.* 2002; Miyazaki *et al.* 2005). Considering the fact that ergatoids cannot start new colonies without nestmate workers (Murakami *et al.* 2000), their morphologies may be specialized to exploit the reproductive strategy of colonial fission, which is favourable to environments with patchily distributed nest sites (Ohkawara *et al.* 1993; Murakami *et al.* 2002). In this regard, although ergatoids are unable to fly, their ability to mate and reproduce sexually is comparable with that of queens. Such correlation between morphological characteristics and environmental conditions appears in other ant species producing wingless reproductives as well (Heinze & Buschinger 1989; Tinaut & Heinze 1992; Peeters & Ito 2001; Molet & Peeters 2006). Thus, investigating caste differences in the development of body parts will allow us to understand the developmental pathways of ergatoids compared with those of the other two castes. In this study, we used histological observations of eye, wing and gonad development among three female castes in *M. nipponica* to compare the derived developmental pathways (ergatoid-worker pathways) with ancestral pathways (queen-worker pathways). The results suggest that the modular modification of post-embryonic development might promote the phenotypic evolution of novel castes.

2. MATERIAL AND METHODS

(a) *Ants*

Myrmecina nipponica Wheeler inhabits mosses, fern roots and decaying logs on the forest floor. Ants have a body length of 3–4 mm, and the average colony size is approximately 40 adult individuals (Ohkawara *et al.* 1993; Murakami *et al.* 2002). We sampled colonies from the Tomakomai Experimental Forest of Hokkaido University and natural forests near Chitose City from May to September during 2004

through to 2006. Immediately after collection, some colonies were fixed in 70 per cent ethanol and FAA fixative (formalin : acetic acid : ethanol = 6 : 1 : 16) for morphometric and histological studies, respectively, and the remaining colonies were maintained in the laboratory at room temperature until fixation.

(b) *Morphological differences among castes*

As the larval morphologies of numerous ant species differ markedly depending on developmental fate (Wheeler & Wheeler 1976; Sameshima *et al.* 2004), in *M. nipponica*, these larvae might be classified as either undifferentiating or differentiating into queens, ergatoids, workers or males on the basis of their morphological differences. Therefore, to examine the allometric differences in larvae destined to be reproductives and workers in alate queen and ergatoid colonies, we conducted morphometric studies on 267 and 234 fixed larvae randomly collected from 15 alate queen and 20 ergatoid colonies, respectively. Head and body length were measured using an image analysis system (HIM-1; HOGA, Kyoto, Japan).

(c) *Histological studies*

To study the developmental processes associated with caste differentiation, the developmental characteristics of four primordial tissues in larvae (i.e. gonad primordium and imaginal discs of the eye, forewing and hindwing) were histologically examined in three female castes. These primordia were selected because the adult morphologies of these body parts show caste specificities (Miyazaki *et al.* 2005). Next, we conducted histological examinations on pupal tissues of the compound eyes, wings and gonads.

Samples were fixed in FAA for 12–24 h, transferred to 70 per cent ethanol, and dehydrated in a graded series of ethanol concentrations; the samples were then cleared in xylene and embedded in paraffin. Specimens were cut into 5 µm thick sections, which were dewaxed in xylene and dehydrated through a series of increasing ethanol concentrations. Haematoxylin (0.1% haematoxylin, 0.02% NaIO₃, 5% AlK(SO₄)₂, 0.1% citric acid, 5% trichloroacetaldehyde) and 0.5 per cent eosin were used for staining. A BX-51 microscope (Olympus, Tokyo, Japan) equipped with a DP-50 CCD camera (Olympus) and VIEWFINDER LITE software (Olympus) was used for observations. The areas of the sectioned larval tissues were measured using the IMAGE J software (<http://rsb.info.nih.gov/ij/>), and the volumes were calculated as the total of all areas for a larva. Pupal compound-eye widths were measured using an image analysis system (HOGA). The sectioned pupal wings and gonads were observed and compared among three castes. Male larvae and pupae were discriminated by morphology of gonad primordium and apparent external morphologies, respectively, and were excluded from comparisons among females.

(d) *Statistical analysis*

The bimodalities in primordial tissue sizes tested might represent a differentiation between reproductives and workers in each colony type. To detect bimodality in the measurement set for each tissue size during both larval and pupal periods, we conducted a parametric bootstrap test based on statistical models using a finite Gaussian mixture distribution (McLachlan & Peel 2000). The statistical procedure consisted of two steps: model fitting and a bootstrapping test. All data for the primordial sizes were independently fitted by both unimodal (Model 1) and bimodal (Model 2)

models, which were expressed by a Gaussian distribution and two Gaussian mixture distributions, respectively. In each model fitting, the goodness of fit was evaluated based on log likelihood and all parameters were estimated such that the log likelihood was maximized (cf. Leish 2004). We also evaluated deviance, which was just equal to -2 times maximized log likelihood, to compare between Models 1 and 2 in accordance with statistical custom. Here, we defined the difference between deviances (DD), DD_{obs}, for a sample set as the deviance of Model 2 minus the deviance of Model 1. For the parametric bootstrap test, we independently generated 1000 sets of random numbers following the bimodal distribution of which parameters were estimated by fitting Model 1 to the focal measurements. The sample size of each set in the test was equal to the number of the focal measurements. Subsequently, we evaluated DD for all sets to determine a probabilistic distribution of DD under a null hypothesis stating that Model 1 was true. We rejected the null hypothesis when DD_{obs} was larger than the 95 per cent quantile, DD₉₅, of the DD distribution. The estimates for the Gaussian mixture distributions were obtained using FLEXMIX 2.2-3 (Leish 2004) on R-2.9.2 (R Development Core Team 2009).

In addition, a principal component analysis was performed using measurements of all four larval primordial tissues, and then allometric differences among castes were tested at the larval stage. The statistical program SSSMCTH2 (ESUMI, Tokyo, Japan) was used for the principal component analysis.

3. RESULTS

(a) *Size differentiation between reproductive and worker larvae*

In general, as ant larvae undergo several moults, instars can be discriminated by the larval head size. In *M. nipponica*, therefore, three larval instars were identified on the basis of head length (figure 2a). The mean body lengths ± s.d. of the first, second and third instar larvae were 0.68 ± 0.12, 1.00 ± 0.15 and 1.70 ± 0.36 mm in alate-queen colonies, and 0.66 ± 0.10, 0.97 ± 0.13 and 1.69 ± 0.36 mm in ergatoid colonies, respectively (figure 2b). The distributions of larval body lengths in both first and second instars were unimodal in both alate queen and ergatoid colonies (data not shown). Although the body lengths in third instar larvae deceptively showed bimodal frequencies in both colonies (electronic supplementary material, figure S1), larger groups were corresponding to prepupae (data not shown). These results suggest that no size differentiation occurs between queen/ergatoid larvae and worker larvae, as opposed to the adult size differences among the three castes (Miyazaki *et al.* 2005). Statistical analysis showed that for all larval instars, no significant difference in body length was found between the two colony types (Student's *t*-test: *p* = 0.53, 0.21 and 0.79 for first, second and third instars, respectively), indicating that there was no difference in larval body size between queen larvae and ergatoid larvae.

(b) *Queen-worker differentiation of imaginal primordia during larval stage*

As the larval morphological differences among castes were expected to be the highest in the latter stage of the

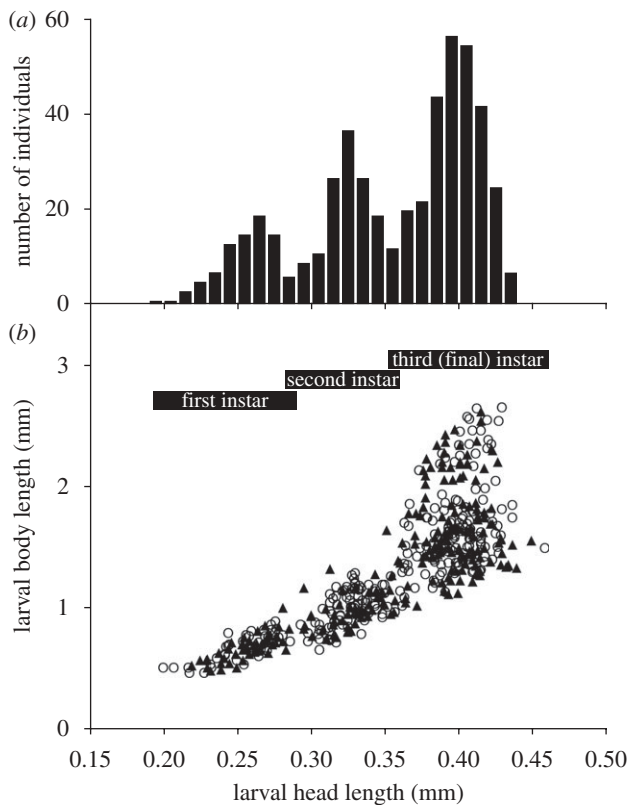


Figure 2. Morphometric analyses of the head and body length in 501 larvae (267 larvae collected from 15 alate-queen colonies and 234 larvae collected from 20 ergatoid colonies). (a) Frequency distribution of larval head length, in which three larval instars were recognized. (b) Scatter plots of larval body length versus larval head length. Open circles, larvae collected from alate-queen ($n = 267$); black triangles, from ergatoid colonies ($n = 234$), respectively. Black bars indicated ranges of larval head length in each larval instar. Note the conspicuous increase in body length in the third instar. No caste-specific allometric growth was detected.

third larval instar, larvae with body lengths of 1.7–2.7 mm (cf. figure 2) were selected for histological analysis. However, prepupae in which imaginal discs had already evaginated were excluded from this analysis. Imaginal primordia on the histological sections were observed in detail (figure 3) and the volumes were calculated (table 1). If queen-worker differentiation already occurred at this larval stage, caste fates of these larvae could be discriminated and the parametric bootstrap tests were performed. As a result, volumes of the forewing disc, hindwing disc and gonad primordium in larvae derived from alate-queen colonies exhibited a bimodal distribution (electronic supplementary material, table S1 and figure 3*g–i*). Each primordium, however, showed different grouping patterns. As the bootstrap test showed that hindwing disc bimodality was more likely than in other primordia (cf. maximized log likelihood in the electronic supplementary material, table S1), the caste fates of larvae from alate-queen colonies were determined on the basis of the hindwing disc volumes; six larvae with larger hindwing discs were thought to differentiate into queens (queen larvae; black bars in figure 3*f–i*), and the remaining 18 larvae with smaller hindwing discs were differentiating into workers (worker larvae; white bars in

figure 3*f–i*). The statistical grouping for forewing disc volumes was similar to the grouping for hindwing disc volumes (figure 3*g*), i.e. queen larvae had significantly larger forewing discs than did the worker larvae (table 1; Student's t -test: $p < 0.001$). This finding suggests that queen differentiation would have already started in the forewing discs at this stage also. The statistical grouping for gonad primordium volumes, however, was different from the grouping for both wing disc volumes. Thus, the bimodality of gonad primordium volume was considered not to represent the caste fates. In queen larvae, however, gonad primordia and eye discs, in which bimodality was not confirmed (figure 3*f* and the electronic supplementary material, table S1), were significantly larger than in worker larvae (table 1; Student's t -test: $p < 0.05$ and $p < 0.001$, respectively), suggesting that queen differentiation in these two primordia would start just before this stage. Therefore, although no caste differences in larval body size could be discerned, the developmental degrees of all four primordia showed that queen differentiation would have started by at least the latter stage of the third instar.

(c) Ergatoid-worker differentiation of imaginal primordia during larval stage

Although larvae from ergatoid colonies potentially develop into future workers and ergatoids, bimodal distributions were not detected in the distributions of any of primordium volumes by the bootstrap tests (electronic supplementary material, table S1 and figure 3*j–m*). Compared with the worker larvae from alate-queen colonies, the sizes of forewing discs and gonad primordia were not significantly different (table 1; Student's t -test: $p = 0.53, 0.61$), although the eye and hindwing discs of ergatoid colony larvae were significantly larger (table 1; Student's t -test: both $p < 0.05$). These results suggest that third instar larvae in ergatoid colonies would include larvae that started differentiating into ergatoids. In two individuals of larvae, eye disc volumes were approximately $5 \times 10^3 \mu\text{m}^3$, which was greater than the eye disc volumes of worker larvae in alate-queen colonies (white bars in figure 3*f*) and comparable with those of queen larvae (black bars in figure 3*f*). Moreover, in four larvae, hindwing discs measuring approximately $0.9–1.2 \times 10^5 \mu\text{m}^3$ were larger than hindwing discs of worker larvae in alate-queen colonies (white bars in figure 3*h*) and comparable with those of queen larvae (black bars in figure 3*h*). One of these larvae had both larger eye and hindwing discs. In addition, a principal component analysis revealed that at least a part of the five larvae with larger eye- and hindwing discs were differentiated from worker larvae (electronic supplementary material, figure S2 and table S2). Therefore, although it was difficult to strictly discriminate ergatoid larvae from worker larvae, these five larvae were likely to differentiate into ergatoids (red bars in figure 3*j–m*, hereafter referred to as a 'putative ergatoid larva').

(d) Differentiation of eyes and ovaries during pupal stage

To examine the developmental processes during the pupal stage, morphological differences of the aforementioned structures were compared among castes. In both colony types, the width of the compound eyes of pupae exhibited

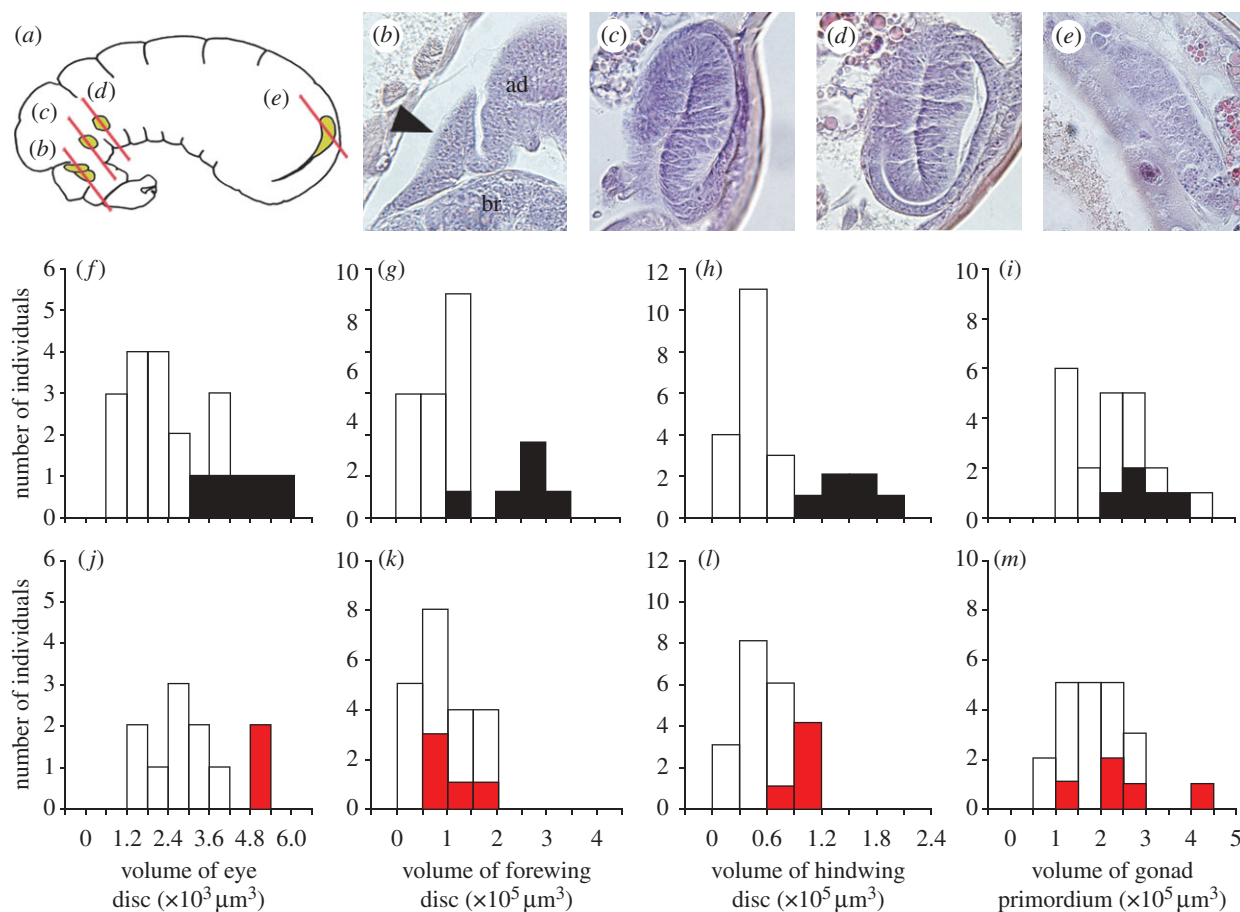


Figure 3. Size differences of four primordia among castes sampled in the latter stage of the third instar larvae. (a) Final instar larvae were sectioned in plane across the primordia (red lines in *a*), and the volumes of the (b) eye (arrowhead), (c) forewing, (d) hindwing disc and (e) gonad primordium were calculated by integrating section areas (ad, antennal disc; br, brain). (f–i) Histograms for sizes of these primordial tissues from larvae collected from alate-queen colonies. Queen and worker larvae were discriminated by a parametric bootstrap test for hindwing disc volumes and are shown as black and white bars, respectively. (j–m) Histograms for primordial tissue sizes of larvae collected from ergatoid colonies. Five larvae (red bars) were defined as putative ergatoid larvae based on their eye and hindwing disc sizes. In three of the five putative ergatoid larvae, the data for eye disc sizes were lacking, because of a technical reason (§2).

Table 1. Volumes of four primordial tissues: imaginal eye disc ($\times 10^3 \mu\text{m}^3$), forewing disc ($\times 10^5 \mu\text{m}^3$), hindwing disc ($\times 10^5 \mu\text{m}^3$) and gonad primordium ($\times 10^5 \mu\text{m}^3$) in late third instar larvae collected from alate-queen and ergatoid colonies. (Among larvae collected from alate-queen colonies, queen larvae could be discriminated from worker larvae based on wing disc volumes. All data are shown as mean \pm s.d.)

	larvae from alate-queen colonies			larvae from ergatoid colonies
	all larvae	queen larvae	worker larvae	
eye disc	2.67 \pm 1.40 (<i>n</i> = 20)	4.23 \pm 1.04 (<i>n</i> = 6)	2.01 \pm 0.94 (<i>n</i> = 14)	3.10 \pm 1.24 (<i>n</i> = 11)
forewing disc	1.26 \pm 0.80 (<i>n</i> = 24)	2.38 \pm 0.64 (<i>n</i> = 6)	0.89 \pm 0.40 (<i>n</i> = 18)	0.97 \pm 0.46 (<i>n</i> = 21)
hindwing disc	0.71 \pm 0.49 (<i>n</i> = 24)	1.46 \pm 0.29 (<i>n</i> = 6)	0.46 \pm 0.18 (<i>n</i> = 18)	0.62 \pm 0.27 (<i>n</i> = 21)
gonad primordium	2.26 \pm 0.82 (<i>n</i> = 22)	2.98 \pm 0.69 (<i>n</i> = 5)	2.04 \pm 0.74 (<i>n</i> = 17)	1.90 \pm 0.84 (<i>n</i> = 21)

a bimodal frequency distribution (parametric bootstrap test, figure 4*a,b* and the electronic supplementary material, table S1), indicating that it is possible to discriminate among queen (black bars in figure 4*a*), ergatoid (red bars in figure 4*b*) and worker pupae (white bars in figure 4*a,b*) on the basis of compound-eye width (table 2). In adults, the compound-eye size is a critical cue for the caste discrimination (Murakami *et al.* 2002; Miyazaki *et al.* 2005). The compound-eye width of ergatoid pupae was significantly smaller than that of queen pupae (Student's *t*-test: $p < 0.001$; table 2).

Insect ovaries generally consist of several ovarioles, and ovariole number is often used as an index of potential fecundity. The mean number of pupal ovarioles \pm s.d. was 10.6 \pm 1.3 in queen pupae (*n* = 8), 10.3 \pm 1.6 in ergatoid pupae (*n* = 9) and 7.8 \pm 3.7 in worker pupae (*n* = 6). Although no significant difference was observed in mean ovariole number among the three castes (figure 4*c*; Kruskal–Wallis test: $p = 0.23$), the variance in ovariole number was significantly greater in workers than in queens and ergatoids (Bartlett test: $p < 0.05$). These findings suggested that the caste differences in

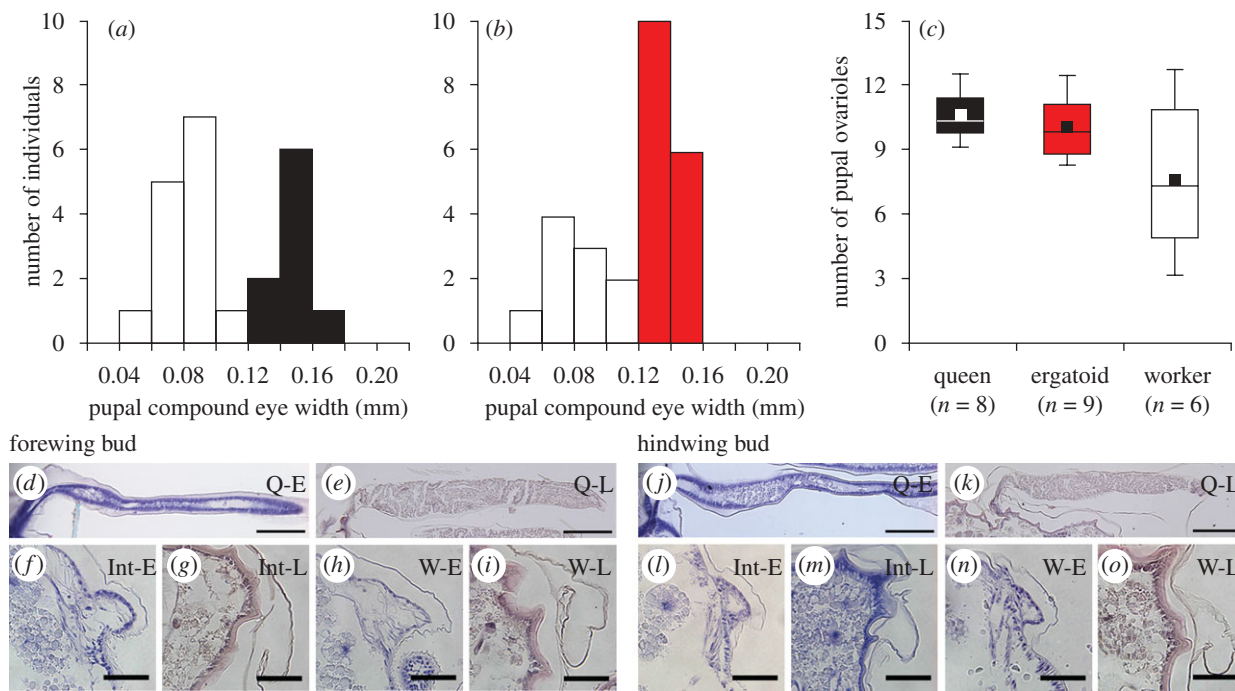


Figure 4. Morphological differences among castes at the pupal stage. (a,b) Frequency distributions of pupal compound-eye width in (a) alate-queen and (b) ergatoid colonies. Queen and ergatoid pupae (black and red bars, respectively) were discriminated from worker pupae (white bars). (c) Numbers of pupal ovarioles were compared among queen, ergatoid and worker pupae (black, red and white boxes, respectively). The upper lines of the boxes indicate the 75th percentile, the bottom edges indicate the 25th percentile, and the centrelines indicate the median. The bars above and below the boxes indicate 90th and 10th percentiles, respectively, and the squares show the average. (d–i) Forewing bud, (j–o) hindwing bud. (d–o) Transverse sections of pupal wing buds in each caste at the early and the late pupal stages. In queen pupae, the well-developed wing buds of the forewings and hindwings were filled with double-layered epithelial tissue at the early stage (d,j), but this epithelium became multi-folded at the late stage (e,k). Ergatoid and worker pupae had two pairs of wing buds, which were much smaller than those of queen pupae. These wing buds were filled with immature epithelial tissue at the early stage (ergatoid: f,l; worker: h,n). The epithelium inside the wing buds disappeared by the late stage (ergatoid: g,m; worker: i,o). Scale bars, indicate (d,e,j,k) 200 μ m and (f–i,l–o) 50 μ m. Abbreviations of each panel indicate the caste fate and stage: Q, queen; Erg, ergatoid; W, worker; E, early pupal stage; L, late pupal stage.

Table 2. Widths of pupal compound eyes in both colony types. (Reproductive and worker pupae were discriminated by the parametric bootstrap tests in each colony type. All data are shown as mean \pm s.d.)

	from alate-queen colonies		from ergatoid colonies	
	queen pupae	worker pupae	ergatoid pupae	worker pupae
pupal compound-eye width	0.15 \pm 0.01 (n = 9)	0.08 \pm 0.01 (n = 18)	0.13 \pm 0.01 (n = 16)	0.08 \pm 0.02 (n = 10)

ovaries between reproductive castes and workers would become obvious, probably because the ovarioles of workers degenerated during pupal development.

(e) Wing development during pupal stage

To observe wing development in detail, the pupal stage was divided into early and late stages on the basis of the degree of adult cuticle formation. The adult cuticle is produced under the pupal cuticle during pupal development and is completed only during the late pupal stage. In the early stage, queen pupae had two pairs of well-developed wing buds. These wing buds initially consisted of undeveloped epithelial tissues (figure 4d,j), which became multi-folded by the late pupal stage (figure 4e,k). In queens, wing formation is generally achieved throughout post-embryonic development. Interestingly, ergatoid and worker pupae had two pairs of small wing buds that

contained undeveloped epithelia (figure 4f,h,l,n). However, these tissues appeared to have degenerated and contained haemolymph by the late pupal stage (figure 4g,i,m,o). These observations suggest that in workers and ergatoids, wing discs were once evaginated at pupation but degenerated later, during the late pupal stage. Although mesothoracic structures specific to adult ergatoids, i.e. small forewing buds and small foveae or wing processes, were sometimes observed (figure 1b inset; Miyazaki *et al.* 2005), the developmental processes forming these structures were not examined in this study.

4. DISCUSSION

This study showed that the developmental processes forming adult body parts associated with caste development differed among three female castes, i.e. queen, ergatoid and worker, in *M. nipponica*. In addition, the

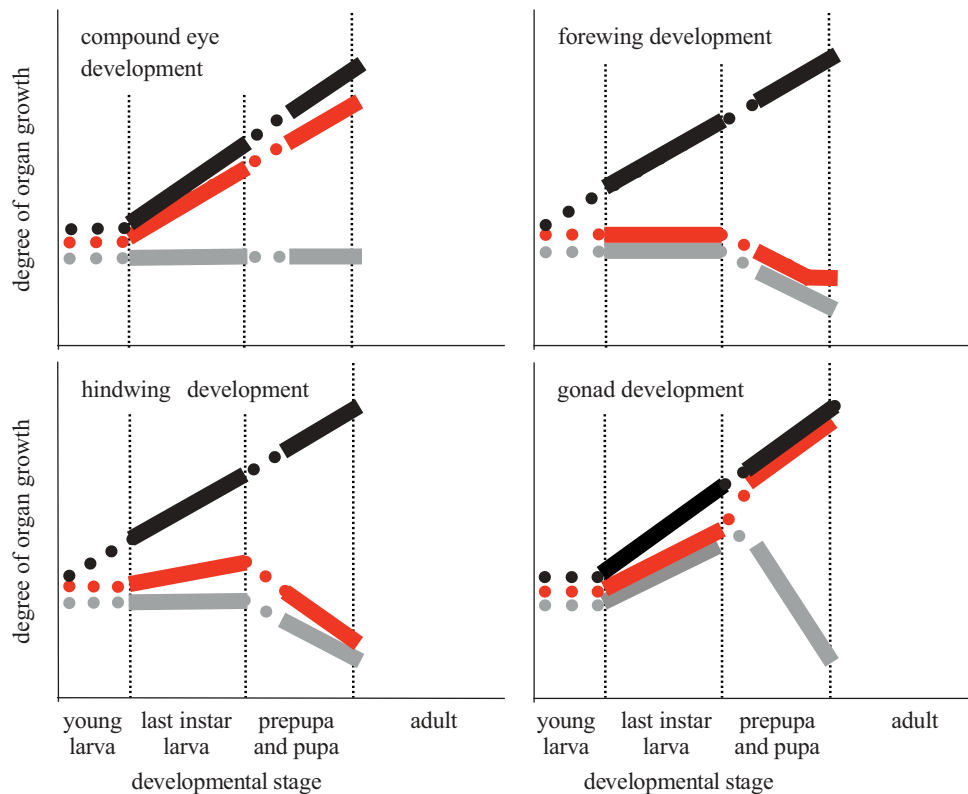


Figure 5. Schematic diagrams of developmental schedules for caste-specific organs. The developmental schedules differ among organs in each caste, suggesting the existence of differential developmental regulations among organs. In ergatoids, the development of compound eyes was regulated in a queen-like manner, and those of forewings and hindwings were regulated in a manner similar to that in workers. Interestingly, gonad development in ergatoids was worker-like during the larval stage and became queen-like at the pupal stage. Black, red and grey lines show the developmental schedules of queen, ergatoid and worker ants, respectively. Solid and dotted lines indicate the observed and expected processes of organ development respectively. Longitudinal and transverse axes show the degree of organ growth and developmental stage, respectively.

developmental regulations underlying the post-embryonic caste differentiation are considered to be specific to body parts, i.e. the eyes, wings and reproductive organs. Comparison of eye disc size in third instar larvae showed that both queen-worker and ergatoid-worker differentiation had already started during this stage (figure 3*f,j*). At the pupal stage, the caste differences in compound-eye sizes were more apparent between reproductive and worker pupae in each colony type (figure 4*a,b* and table 2). In adults, the morphological differences in the compound eyes and ocelli of the two reproductives are distinctive (Miyazaki *et al.* 2005). Considering that ocelli are also derived from eye discs (Held 2002), these differences suggest that the rate of eye disc development would either be slower or the determination of caste fate would be delayed in ergatoid larvae (figure 5).

In ergatoids, the gonad primordia develop slowly during the larval period, as in worker larvae, but develop dramatically during the pupal period, as in queen pupae. This developmental switching might be the result of resource allocation at a prepupal stage because some larval tissues degenerated during the prepupal and pupal periods (Chapman 1998). Similar resource allocations are found in many ant species, in which flight muscles degenerate in dealate queens to allocate energy to reproductive organs (Hölldobler & Wilson 1990).

In most ant species, wings are designated as one of the queen-specific characteristics. In alate-queen colonies of

M. nipponica, queen differentiation of wings had already started during the larval period (figure 3*g,h*), and the wing buds of queen pupae were particularly well developed (figure 4*d,e,j,k*). On the other hand, small wing buds appeared once in worker pupae in the early pupal stage but degenerated by the time the adult cuticle formed (figure 4*h,i,n,o*). Wing development in ergatoids was similar to that in workers, with minor differences. Although the forewing discs in ergatoid larvae were similar to those in worker larvae (figure 3*k*), their hindwing discs were larger than those in worker larvae but not as large as those in queen larvae (figure 3*l*). At the pupal stage, however, the developmental processes of both wings were quite similar to those in worker pupae (figure 4*f,g,l,m*). In adult ergatoids, unique structures such as thoracic projections or small foveae were often observed at the site of the forewing attachment, and no structures were observed at the hindwing position (Miyazaki *et al.* 2005). In conclusion, wing development in ergatoids may be regulated in a worker-like and ergatoid-specific manner depending on the forewing/hindwing development and the developmental stages. As in other ant species (Sameshima *et al.* 2004; Gotoh *et al.* 2005), this degenerating process would be mediated by apoptosis.

As far as we know, this is the first study in which the developmental pathways of multiple caste-specific traits were examined. This study also gives us evolutionary implications concerning the modification processes of

caste developmental pathways. Other ant species have secondarily evolved castes, and their developmental pathways appear to be a modification of the ancestral pathways used to produce either queens or workers (Peeters 1991). In *M. nipponica*, ergatoids possessed developmental patterns distinct from those of queens and workers. Interestingly, the differentiation patterns of ergatoids varied depending on body parts, and followed different developmental trajectories in a modular manner; some body parts showed queen-like patterns (figure 1*d*) and others showed worker-like patterns (figure 1*e*). On the basis of these findings, ergatoids appear to be mosaics between queens and workers, although some body parts developed in an ergatoid-specific manner (figure 5). As the timing of body-module development/growth differed among castes, these developmental mechanisms can be regarded as a heterochronic developmental regulation (Gould 1977; Hall 1999; West-Eberhard 2003). In addition, it was thought that such modular developmental regulations would play an important role in the evolution of some peculiar ant castes.

Although the evolutionary origin of ergatoids was not explained in this study, a novel developmental pathway for ergatoids is suggested to have acquired through 'genetic accommodation' (West-Eberhard 2003). Previous studies report two important findings: (i) ergatoids have been described only in low-temperature localities and can be experimentally induced by a low-temperature condition and (ii) the phenotypes of female reproductives follow the preceding generation. Therefore, ecological stresses such as low temperature, low food resources or patchily distributed nest sites may be responsible for developmental modifications to pre-existing queen-worker differentiation pathways. In general, environmental stresses can relax the canalization of developmental pathways (Hall 1999). In the case of *Apis mellifera*, the environmental stresses, such as manipulations of larval diet and endocrinal condition, produce a variety of intermorphs with some worker-like traits and some queen-like traits (reviewed in West-Eberhard 2003). Considering these facts, in *M. nipponica*, the canalized developmental pathway to queens may have been relaxed by the aforementioned stresses, resulting in a variety of mosaic queen phenotypes. Subsequently, the ergatoid reproductives would become adaptive by means of the new reproductive strategy, i.e. colony fission, under harsh ecological conditions where nuptial flights would be disadvantageous (Peeters & Ito 2001). Importantly, although wings and flight muscles, which require high energy costs for the development, are not necessary for mating of wingless reproductive castes, the development of eyes and gonads may be necessary for colony fission (dispersal on ground) and sexual reproduction, respectively (Ohkawara *et al.* 1993; Murakami *et al.* 2000, 2002). Therefore, the novel bifurcated developmental pathways would have subsequently become fixed through genetic assimilation (or genetic accommodation) in some lineages inhabiting cold regions.

Thus far, *M. nipponica* ergatoids have been reported from two localities located more than 1000 km apart from each other (Murakami *et al.* 2002), although their evolutionary origins within the species remain unknown. Furthermore, wingless reproductive castes like ergatoids

have frequently been described in the genus *Myrmecina* (Ito 1996; Buschinger & Schreiber 2002; Steiner *et al.* 2006). Although the molecular phylogeny among species in the genus was recently proposed (Steiner *et al.* 2006), the evolutionary origins of wingless reproductives among species remain debatable. Future research will include comparative developmental analyses to reveal how the caste differentiation pathways have evolved.

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