Juvenile Hormone titre and vitellogenin gene expression related to ovarian development in primary reproductives, as compared to nymphs and nymphoid reproductives of the termite *Reticulitermes speratus*

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Abstract

To elucidate the reproductive cycle of termite queens, incipient colonies of *Reticulitermes speratus* (Isoptera: Rhinotermitidae) were established under laboratory conditions, and the transition of colony development was observed at 0.5, 1.5, 2.5, 3.5, and 7.5 months (stages I–V, respectively) after colony foundation. Ovarian development, vitellogenin gene expression, and Juvenile Hormone (JH) titres were examined in the queens and in non-physogastric nymphoids collected from natural colonies. A reproductive cycle in queens is observed, in which the oviposition rate is relatively higher during stages I and II, then decreases during stages III and IV. Vitellogenic oocytes are not observed in the ovary during the stages III and IV, and the expression level of the vitellogenin gene is low, suggesting that egg production in queens is repressed during these stages. However, vitellogenin gene expression and egg deposition in queens resumes during stage V. Juvenile Hormone levels rise during the transition from nymphs to stage-I queens, and elevated JH titres are observed also during stages III and IV. The decrease in JH titre in queens at stage II preceded the decline in vitellogenesis at stages III and IV. Thus, JH titre and vitellogenesis are correlated in an offset pattern. However, non-physogastric nymphoid reproductives do not have vitellogenic oocytes in their ovaries, and their JH titre is two-fold higher than that of queens, suggesting that elevated JH titre precedes vitellogenesis as in queens.

Key words: termite, queen, nymphoid, juvenile hormone, vitellogenin, ovary, gene expression

Introduction

Termites are eusocial insects with morphologically different individuals, i.e. castes, in a colony: reproductives, workers, and soldiers (Wilson, 1971). In the reproductive caste, there are primary and secondary reproductives. The former are dealate imagoes (king and queen), and the latter are reproductive individuals moulted from nymphs (nymphoids) or workers (ergatoids) without becoming winged alates (Thorne, 1996; Roisin, 2000). The incipient colony developments have been studied in some species (e.g. *Kalotermes flavicollis*, *Cryptotermes brevis*, *Reticulitermes lucifugus*, and *R. hesperus*), and the reproductive cycles of queens were shown in all of them (reviewed in Nutting (1969)). For example, in *R. lucifugus* oviposition commenced 6 or 7 days after the establishment of laboratory colonies, but the queen then stopped laying eggs when the first brood moulted to fourth-instar workers (Nutting, 1969). In termite incipient colonies, primary reproductives may cease laying eggs and perform other parental duties, including trophallactic feeding to the first offspring. After the emergence of workers in a colony, reproductives can devote themselves to egg production (Nalepa, 1994).

Generally in insects, depending on their reproductive cycles, the fat body is stimulated periodically by hormonal activity to produce vitellogenin, and then vitellogenesis occurs in the ovary (Nijhout, 1994). In particular, the Juvenile Hormone (JH) titre in the haemolymph appears to stimulate vitellogenesis (Hartfelder, 2000). Juvenile Hormone is known to induce vitellogenin synthesis in the fat body of *Diploptera punctata*, *Locusta migratoria* and *Rhodnius prolixus* (Nijhout, 1994). However, in termites, it was shown that JH does not always stimulate the vitellogenesis in reproductives. In a termopsid, *Zootermopsis angusticollis*, it is suggested that JH plays a dual role in queens depending on their stage of development; an elevated JH titre may inhibit reproductive processes in immature alates, or stimulate ovarian activity in mature queens (Brent *et al*., 2005). In a rhinotermitid, *R. flavipes*, JH synthesis correlates to the size of corpora allata and to the number of vitellogenic ovarioles in nymphoids, but not ergatoids (Elliott & Stay, 2007). The rates of JH synthesis in pharate ergatoids far exceeds those of pharate workers, pharate presoldiers and soldiers (Elliott & Stay, 2008). In a termopsid, *Hodotermopsis sjostedti*, the JH titre of female alates was higher than in pseudergates (seventh larval instar), nymphs, presoldiers and soldiers, probably in relation to vitellogenesis, although the JH titre of neotenics (secondary reproductives) was the lowest (Cornette *et al*., 2008). Finally, in a kalotermitid, *Cryptotermes secundus*, JH titres do not differ significantly between alates and primary reproductives, and neotenics have significantly higher JH titres than primary reproductives (Korb *et al*., 2009). Ovarian activity was not clearly shown in reproductives of either of these latter species (Cornette *et al*., 2008; Korb *et al*., 2009). Thus, the role of JH for vitellogenesis in termite reproductives is still unclear.

To provide additional comparative data for interpreting the role of JH in termite reproduction, the ovarian development of queens of the rhinotermitid, *Reticulitermes speratus* (Kolbe), was monitored together with quantification of JH titre. The development of incipient colonies, which were established under laboratory conditions, was determined by recoding colony composition at 0.5, 1.5, 2.5, 3.5, and 7.5 months (stages I–V, respectively) after colony foundation. Queens were sampled at each stage to quantify ovarian development, the expression of the vitellogenin gene, and JH titre of those queens. These factors were also examined in non-physogastric nymphoids collected from natural colonies. Based on these results, the role of JH for vitellogenesis in *R. speratus* reproductives is discussed.

Materials and Methods

Termites

Four (A-D) mature termite colonies were collected from Kureha Hill, and one (E) from Furudo, in Toyama Prefecture, Japan, in May and September 2008. Pieces of logs were brought back to the laboratory and kept in plastic cases under constant darkness. Sixth-instar nymphs (N6; Takematsu, 1992; Maekawa *et al*., 2008) with swollen wing buds, just prior to the moult into alates, and nymphoid reproductives were picked from each colony. All nymphoids used here (about 30 individuals collected from colony E) were non-physogastric, and no eggs were found in their nest. Nymphoids were identified by a longer abdomen and darker pigmentation than nymphs (Lainé & Wright, 2003; Maekawa *et al*., 2008).

Colony foundation

After the emergence of alates, the sexes of termite individuals were discriminated according to the morphology of their abdominal tergites (Weesner, 1969). Dealated adults were chosen randomly from each colony and female-male pairs were mated under the following conditions: one female from one colony and one male from another (female from colony A and male from colony B: 49 pairs, AC: 69, AD: 85, AE: 45, BA: 58, CA: 28, DA: 129, and EA: 80). Each pair was placed in a 20 mL glass vial with *c.* 8 g of mixed sawdust food (Mitani, Ibaraki, Japan) and kept at 25°C under constant darkness. Colonies were then sampled after 0.5, 1.5, 2.5, 3.5 and 7.5 months (stage I–V, respectively). For measurements of each colony size (numbers of eggs, larvae, workers, and soldiers) and histological observations of the ovaries (see below), samples were fixed in formaldehyde/ethanol/acetic acid (6:16:1, v/v/v) for at least 24 h and stored in 70% ethanol. For RNA and JH extraction (see below), samples were immersed immediately in liquid nitrogen and

stored at -80°C until use.

Histological observations

To describe the histological characteristics of ovaries in N6 nymphs, alates, primary, and neotenic reproductives, paraffin sections were made and stained with eosin and hematoxylin. Abdomens (from more than 3 individuals for each category) preserved in 70% ethanol were dehydrated in increasing concentrations of ethanol, then transferred into xylene, and finally embedded in paraffin. Serial parasagittal sections (10 µm thick) were processed by an MRS80-074 microtome (Ikemoto, Tokyo, Japan) and stained with eosin and hematoxylin. Numbers of abdomens sectioned were 6 (N6 nymph), 8 (alate), 7 (stage I queen), 4 (stage II-IV queens), 5 (stage V queen), and 3 (nymphoid). Tissues on slides were observed using BX-40 (Olympus, Tokyo, Japan) and BZ-8100 (Keyence, Osaka, Japan) microscopes. Numbers of vitellogenic oocytes were counted in each individual, and these numbers were used to evaluate the degree of ovarian maturation.

cDNA preparation

Total RNA was extracted from individual termites stored at -80°C using a FastPure RNA kit (Takara Bio, Shiga, Japan). Three different individuals were used for each category. For single strand cDNA synthesis, DNase-treated mRNA was transcribed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, California, USA) as instructed by the manufacturer.

Subcloning and sequencing

Using the cDNAs described above, targeted gene fragments were amplified by PCR with a thermal cycler GeneAmp 2400 (Applied Biosystems, Foster City, California, USA) or MJ-Mini (Bio-Rad, Hercules, California, USA). Primers for PCR were designed, based on the published partial *vitellogenin I* sequence of *R. flavipes* (Scharf *et al*., 2005); forward, 5’-CCA AGT TAT GCG AGT CGC AA-3’, reverse, 5’-CAG GGA AGA TCG CGA CTG A-3’. For real-time quantitative PCR analyses, *beta-actin* was chosen as an endogenous control gene showing constitutive expression, because this gene was evaluated as the most reliable reference gene in *R. flavipes* (Scharf *et al*., 2005; Zhou *et al*., 2006). Based on the conserved region among *R. flavipes* and other animals, degenerate PCR primers for cloning of *beta-actin* were also designed; forward, 5’- GGT CGT ACC ACM GGY ATY GT-3’, reverse, 5’- CGG ATG TCR ACG TCR CAC TT-3’. The PCR products were purified using a MagExtractor kit (Toyobo, Osaka, Japan) and subcloned into a pGEM-T vector (Promega, Madison, Wisconsin, USA) and transfected into *Escherichia coli* JM 109 (Takara Bio). Nucleotide sequences were determined using a DYEnamic ET Terminator Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and an automatic sequencer model 373S (Applied

Biosystems). Nucleotide sequence data shown in this study are available in the DDBJ/EMBL/GenBank databases under accession numbers: AB520715 (vitellogenin gene) and AB520714 (beta-actin gene).

Real-time quantitative PCR

The relative quantifications of transcripts were performed using SYBR Green I reagent and a MiniOpticon Real-Time System (Bio-Rad). *Beta-actin* was used as the endogenous control gene. Primers for the target and control genes were designed using Primer Express software (Applied Biosystems); for *beta-actin*: forward, 5'-AGC GGG AAA TCG TGC GTG AC-3', reverse, 5'-CAA TGG TGA TGA CCT GGC CAT-3'; for *vitellogenin I*: forward, 5'-CCT ACA TGC GTT GTT GAT GG-3', reverse, 5'-TGA CGA CTA TGC ACT CCA GC-3'.

JH extraction

Five individuals stored at -80°C were homogenized in 1 ml methanol/isooctane (1:1, v/v) and the homogenate allowed to stand at room temperature for 30 min. Eight replicated samples (= 40 individuals) were prepared for N6, alates and primary reproductives and 5 replicates (= 25 individuals) for nymphoid reproductives. After centrifugation at 13,900 **g** for 5 min, the supernatant was collected and mixed with 10 µg of fenoxycarb (Wako, Osaka, Japan) as an internal standard. The mixture was vortexed and allowed to stand at room temperature for 30 min, before centrifugation at 5,600 **g** for 15 min. The upper isooctane phase was transferred into a new glass vial, and the methanol phase was vortexed and centrifuged at 7,700 **g** for 30 min, and then combined with the isooctane phase. The resulting mixture was stored at -80°C before vacuum drying in a CC-181 centrifugal concentrator (Tomy, Tokyo, Japan). Dried pellets were dissolved in 20 µl methanol. Experimental details were modified from Cornette *et al*. (2008) and Gotoh *et al*. (2008).

Liquid chromatography-mass spectrometry (LC-MS)

From each 20 µl concentrated sample, 5 µl were separated on a 150 × 2 mm i.d. C18 reverse-phased column (YMC-Pack Pro C-18.5 µm, YMC Co. Ltd., Kyoto, Japan) protected by a guard column (YMC-Pack Pro, sphere ODS, YMC Co. Ltd.) with a gradient elution of water/methanol (0-15 min 80-100% methanol, 15-20 min 100% methanol) at a flow rate of 0.2 mL/min, utilizing an Agilent 1100 HPLC system with an autosampler (Agilent Technologies, Santa Clara, California, USA). Mass spectral analysis was performed by electrospray ionization (ESI) in the positive mode on a microTOF-HS (Brucker Daltonik GmbH, Bremen, Germany) under the following conditions: the electrospray capillary was set at 4.5 kV and the dry temperature was 200°C. The nitrogen pressure for the nebulizer was 1.6 bar and the drying gas nitrogen flow-rate was 9 L/min. Quantification of

JH III and fenoxycarb was performed by monitoring the $[M+H]^+$ and $[M+Na]^+$ ions. A calibration curve for JH III (Sigma-Aldrich, St. Louis, Missouri, USA) was plotted using the same internal standard concentration as fenoxycarb in each sample. The JH III titre from each sample was then calculated after analysis of the chromatogram data using QuantAnalysis software (Bruker Daltonics, Bremen, Germany). Results are expressed as ng/mg wet weight.

Statistical tests

A statistical test was performed by usual methods for multiple comparisons (Tukey's test) using the statistical software Mac Statistical Analysis ver. 1.5 (Esumi, Tokyo, Japan). *P* values less than 0.05 are considered significant.

Results

Numbers of eggs in the colony and ovarian developments

Figure 1 shows the numbers of offspring (eggs, larvae, workers, presoldiers and soldiers) (A) and eggs (B) per colony in each stage. Only single a presoldier, which moulted into a soldier, was observed in some colonies at stage III (19/55 colonies: 35%), and one soldier was observed at stage IV in all colonies examined (56/56 colonies: 100%). During stage V, two soldiers were observed in 3 of 10 colonies, and the remaining 7 colonies had only one soldier. During stages III and IV, the numbers of eggs per colony decreased significantly in comparison with stages I and II (Fig. 1B). The ovaries of N6 nymphs contained only immature oocytes lying in rows, and no vitellogenic oocytes were observed (Fig. 2A). Vitellogenic oocytes could be observed in the ovary of swarming alates (Fig. 2B), although they were probably not fully developed oocytes according to their sizes. These oocytes were used for the initial clutches produced after the colony foundation. Vitellogenic basal oocytes were observed in the ovaries of queens at stages I, II, and V (Figs. 2C, 2D and 2G), when many eggs were found in their colonies (Fig. 1B). At stages III and IV, vitellogenic oocytes were observed rarely in the ovaries of queens (Figs. 2E and 2F). The mean number of vitellogenic oocytes (mean ± S.D.) per queen were 0 (n = 4, stage III) and 0.3 ± 0.5 (n = 4, stage IV), respectively. At stage V, vitellogenic oocytes were observed again in the queen ovaries (Fig. 2G). The degree of ovarian maturation was not so different among alates and queens at stages I, II and V. Indeed, the mean number of vitellogenic oocytes (mean ± S.D.) per queen at stage V (3.4 ± 2.7, n = 5) was similar to those of alates (4.5 ± 0.9, n = 8), stage I (2.4 ± 0.9, n = 7) and stage II (1.3 ± 0.5, n = 4). Non-physogastric nymphoid reproductives had only pre-vitellogenic oocytes in their ovaries (Fig. 2H).

Vitellogenin gene expression

Results from real-time quantitative PCR experiments showed that the expression levels of the vitellogenin gene were quite different among stages of colony development (Fig. 3). For each gene (vitellogenin and beta-actin), a single peak on the melting curve was observed, showing that target PCR products were amplified selectively and that primer dimers and other products were not formed. The vitellogenin gene showed higher expression levels in alates and queens at stages I, II, and V, and lower expression levels among N6, nymphoids, and queens (stages III and IV), although the relative expression levels did not differ significantly among alates and queens at stage I, II, and V (Tukey's test, $p<0.05$) (Fig. 3). The expression level was quite low in N6 nymphs (10^4-fold lower than the level observed in alates), corresponding to a complete lack of vitellogenesis (Fig. 2A), whereas, vitellogenin gene expression was higher during stages I and II, when some vitellogenic oocytes were observed (Figs. 2C and 2D). All individuals examined at these stages showed detectable expression levels of vitellogenin gene. However, the gene expression levels dropped markedly to lower or undetectable levels at the stages III and IV, and then increased at stage V. The expression levels of the vitellogenin gene in nymphoids were significantly lower than those in the queens at stage II.

JH titre quantification

The LC-MS results showed that JH III was the only JH homologue found in *R. speratus* (data not shown). The JH levels were shown to increase from N6 to queens at stage I. Then, a decrease in JH titre in queens at stage II was observed, and relatively high JH titres were maintained during stages III and IV. The highest JH levels were observed in nymphoids, where titres were more than double those in queens at any stages (Fig. 4).

Discussion

Reproductive cycles of queens

In *R. lucifugus*, young queens begin oviposition 6 or 7 days after the colony foundation and lay an initial clutch of 10-20 eggs within a month (Nutting, 1969). Then, the queens stop laying with the hatching of the first larvae about 28 days after the first oviposition. Oviposition resumes 4-6 months later after the first clutch of larvae moult to the fourth instar (workers) (Nutting, 1969). When 30-50 workers are present in a colony, the king and queen cease foraging and rely on their offspring for nutrients (Buchli, 1958; Nutting, 1969). Similar behavioural changes in reproductives are observed in incipient colonies of *R. speratus* under the laboratory conditions in the present study. Colonies contain about 20 individuals at 2.5–3.5 months after colony foundation (stage IV), although eggs

and vitellogenetic oocytes are not observed at stage IV. Queens probably stop laying eggs after stage III, and do not resume oviposition until worker number exceeds a threshold sufficient to support the production of the next brood. This appears to happen at some point prior to 7.5 months, when the average colony contains 30 workers. Queens may cease laying eggs to perform other parental duties, such as trophallactic feeding for their first broods. As the offspring develop, third and higher instars are nutritionally independent of adults and they begin taking brood care (Nalepa, 1994). Numbers of vitellogenic oocytes observed in queens at stage V are similar to those of queens at stages I and II, suggesting that the numerous eggs observed in a colony at the stage V (38.1 ± 15.6, *n*=10; Fig. 1B) are probably produced progressively by the queens at frequent intervals after the reproductive resumption. This also suggests that it takes long time for an egg to mature allowing for an accumulation of eggs in a colony.

Vitellogenin gene expression changes of queens

In *Z. angusticollis*, secondary neotenic reproductives show a high rate of JH III production by the paired corpora allata on 4 days after the neotenic moult (Greenburg & Tobe, 1985). Moreover, haemolymph vitellogenin is detectable at 7-9 days, and oviposition starts at approximately 25 days after the neotenic moult (Greenburg *et al*., 1978). In the lubber grasshopper *Romalea microptera*, virgin female imagos show increased levels of haemolymph JH III in addition to the high vitellogenin-mRNA levels in the fat body, that then drop to undetectable levels on the day of oviposition when vitellogenesis has ceased (Borst *et al*., 2000). Thus, the levels of these two factors, i.e. JH III and vitellogenin, are probably related mutually to one another during the reproductive cycle. Real-time PCR analyses in this study show that ovarian development and vitellogenin gene expression change in parallel throughout the reproductive cycle of *R. speratus* queens. The present results suggest that vitellogenin expression rises markedly in the fat body soon after imaginal moult, and that regulation of the vitellogenin gene expression in the fat body is related strongly to the reproductive cycle and vitellogenesis of *R. speratus* queens.

Changes in JH titre after the imaginal moult

Cornette *et al*. (2008) showed that the titre of JH III in the haemolymph of *H. sjostedti* female alates are the highest among the reproductive castes in this species. These authors suggest that the high JH titre of female alates is probably related to the initiation of egg production. Similarly, Korb *et al*. (2009) show that JH III titre in the haemolymph increased after the imaginal moult compared with the preceding nymphal instar in *C. secundus*. In the present study, the JH titre of N6 nymphs in *R. speratus* is low (*c*. 1.6 ng/mg), and rises modestly at stage I (*c*. 4.6 ng/mg), which is about 3-fold higher than the level of N6. The increase in JH titre is correlated positively with the high levels of vitellogenin expression in queens at stages I and II. Then, the decrease in JH titre in queens at stage

II precedes the decline in vitellogenesis at stages III and IV. In many insects, JH has a stimulatory effect on the vitellogenin expression in fat body tissue (Nijhout, 1994; Hartfelder, 2000). In *R. speratus*, the increase in JH III titre is also correlated strongly with vitellogenin expression in addition to the actual vitellogenesis in the queen ovaries. Unfortunately, JH titre of queens at stage V could not be determined in the present study, because of a shortage of samples. Further studies with more detailed sampling throughout the period covering the stages II–V, in addition to that JH titre and vitellogenin expression should be examined from the pause to resumption of egg production.

Vitellogenin gene expression and JH titre in nymphoid reproductives

Nymphoid reproductives used in this study are non-physogastric, and there are no vitellogenic oocytes in their ovaries. In neotenics, the vitellogenin expression is lower than that of alates, and the ovarian vitellogenesis may not have occurred. However, the JH titre is found to be the highest among any other developmental stages. Thus, shortly thereafter, vitellogenin expression is suggested to be promoted and active vitellogenesis to occur. In the current study, JH was extracted from the homogenized whole insect bodies, thus we are not able to distinguish the haemolymph JH from that derived from other tissues (e.g. fat body or ovary). Namely, there is a possibility that the JH in the haemolymph is taken-up into other tissues in *R. speratus* neotenics, and that a regulatory factor for JH transportation to the specific tissues, such as fat body or ovary, is operated actively for the ovarian development in neotenics. The present results, show neotenics with extraordinarily high JH titres, which accord with those of *R. flavipes* (Elliott & Stay, 2008) and *C. secundus* (Korb *et al*., 2009), but not in *H. sjostedti* (Cornette *et al*., 2008). Korb et al. (2009) and Cornette et al. (2008) analyzed JH titres in haemolymph, but ovarian developmental states of both neotenics at time of JH sampling are not clearly shown. However, neotenics of *H. sjostedti* are producing eggs (Cornette *et al*., 2008). Thus, one explanation for the high titres of JH found in neotenics of *C. secundus* (Korb *et al*., 2009) and in *R. speratus* in the present study, is that development of both neotenics is not completed at the time of sampling and that high JH titres hold them in a pre-vitellogenic state in their natal nests. Further research on the relationship between the JH titre in the haemolymph and vitellogenin expression in each neotenic (non-physogastric and physogastric) are needed to understand the role of JH in vitellogenesis of termite neotenics.

Conclusions

The reproductive cycle in queens of *R. speratus* under laboratory conditions has been observed. The increase in JH III titre is correlated with the transcription of vitellogenin gene and vitellogenesis in ovaries at least after the imaginal moult. In nymphoids, however, high JH titre is inconsistent with the ovarian developmental state. Vitellogenesis and ovarian development could be influenced by

other factors, such as ecdysteroids. Ecdysteroid titres in the haemolymph of alates and queens were investigated in *Z. angusticollis* and *C. secundus* (Brent *et al*., 2005; Korb *et al*., 2009). Both authors show that the relative low level of ecdysteroid titres in queens. However, ecdysteroid titres in the haemolymph of neotenics have not been analyzed in any species. Further analyses on the crosstalks between JH and ecdysteroid (and/or other factors, such as insulin signaling (Wu and Brown, 2006)) are required to understand the whole mechanisms on the ovarian development in termite reproductives.

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Figure caption

Fig. 1

Mean number (mean ± S.D. values) of colony members (including eggs, larvae, workers, presoldiers, and soldiers) per colony (A) and eggs per colony (B) at 0.5, 1.5, 2.5, 3.5, and 7.5 months (stages I–V, respectively) after colony foundation. The numbers of examined colonies are indicated in parentheses. Different letters (a-d) over the bars denote significant differences (Tukey's test, $p<0.05$).

Fig. 2

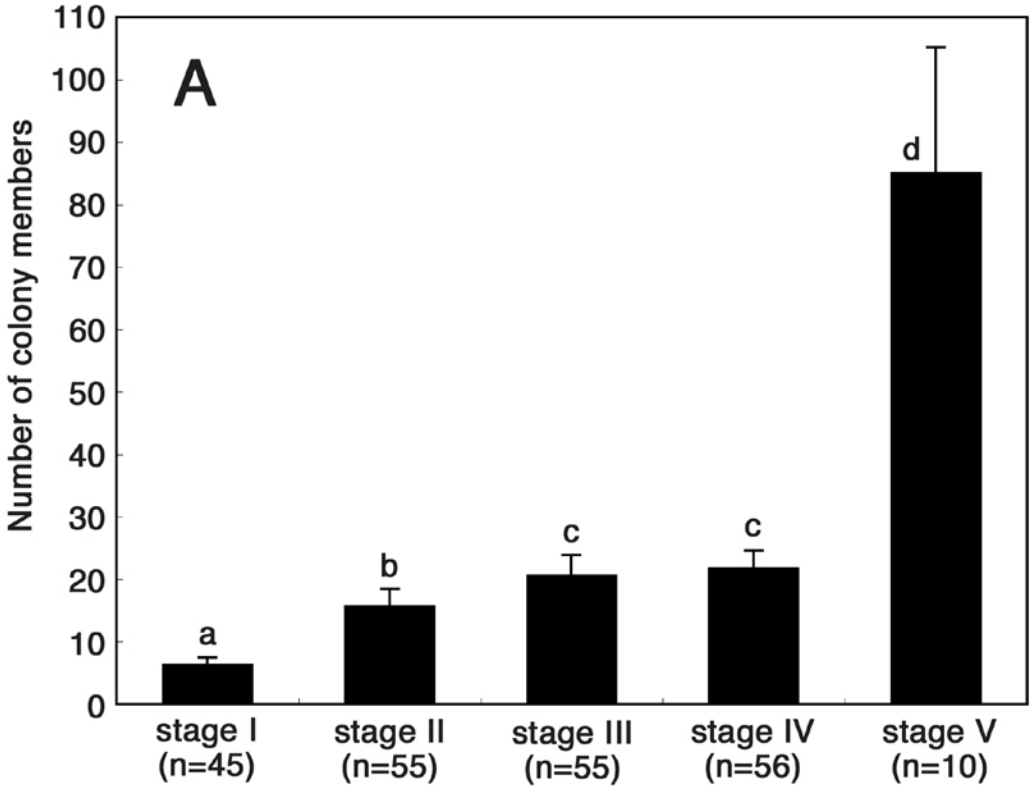
Parasagittal sections of abdomens at each developmental stage (A: N6, B: alate, C: stage I, D: stage II, E: stage III, F: stage IV, G: stage V, H: nymphoid). Vitellogenic basal oocytes were observed in the ovarioles of alates and queens at stages I, II, and V (arrowheads). Each bar indicates 100 μm.

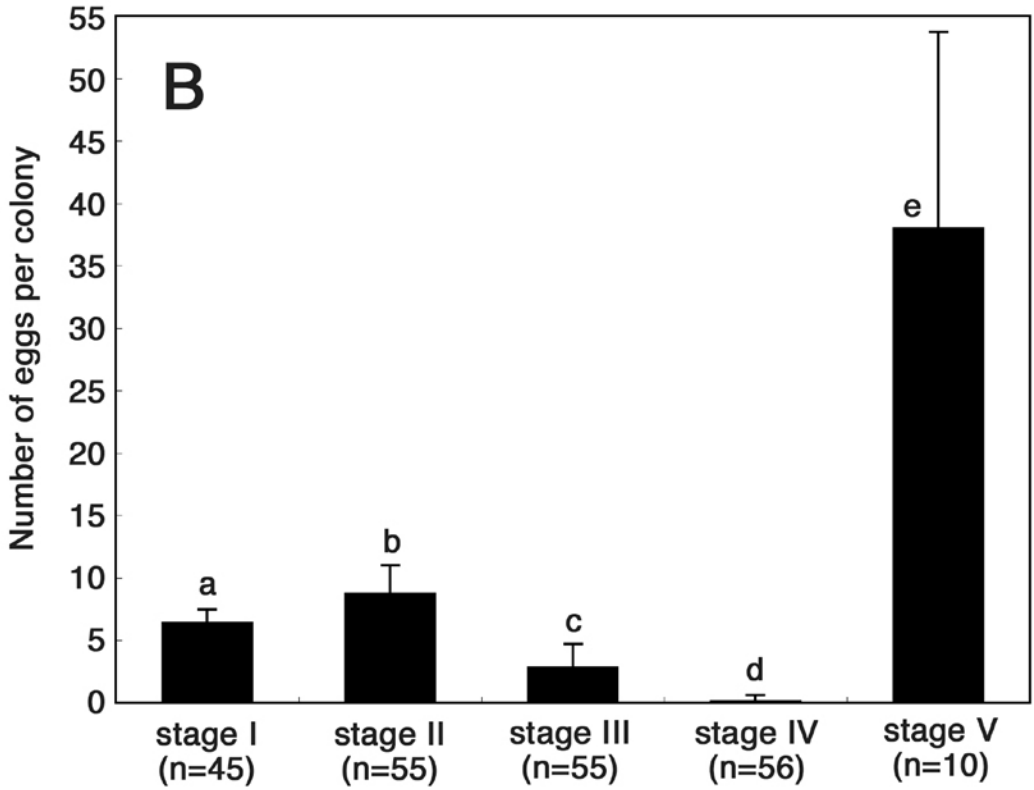
Fig. 3

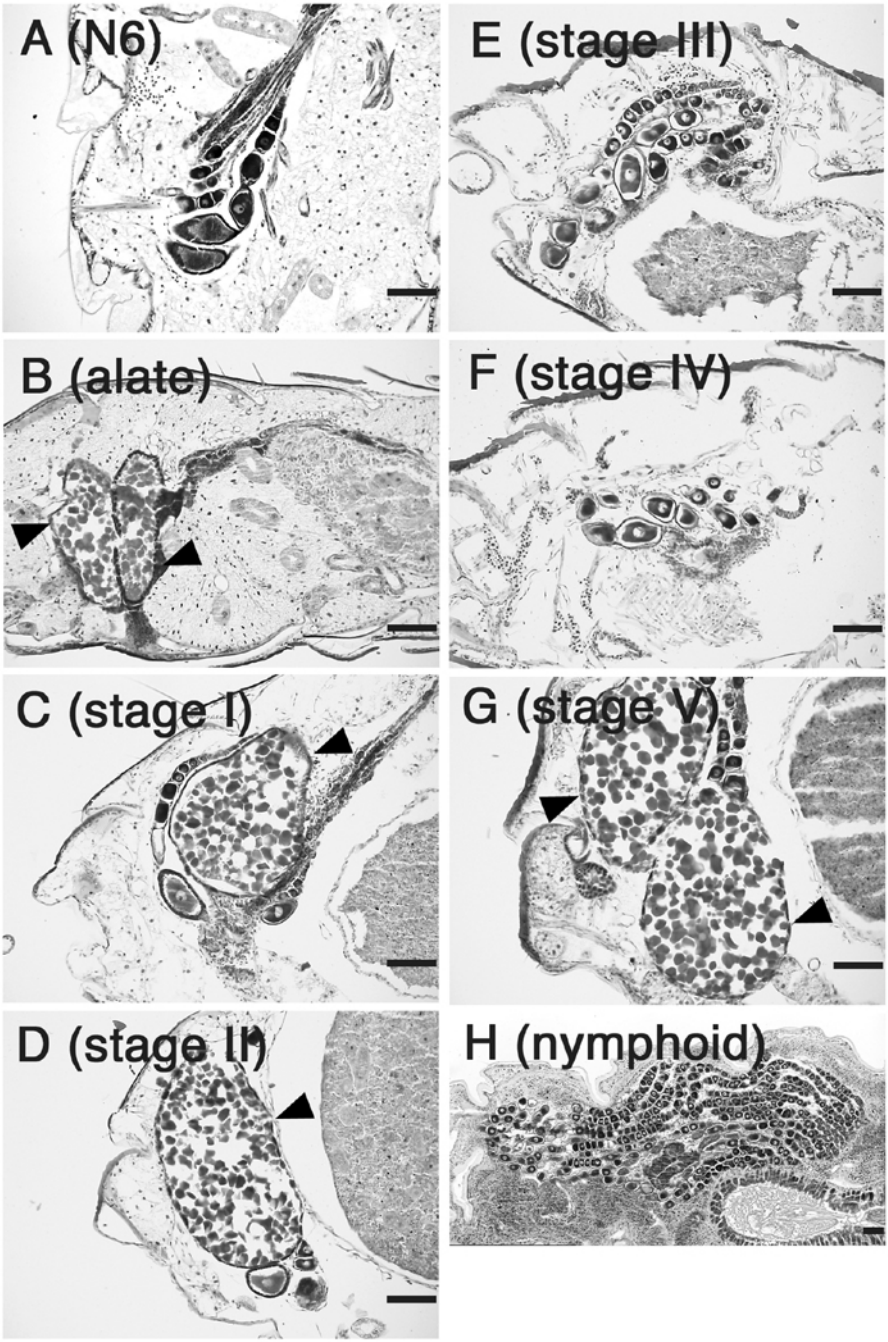
Expression levels (mean ± S.D., $n = 3$) of the vitellogenin gene analyzed by real-time quantitative PCR. The relative expression levels were calibrated using the mean expression level of alates as 1.0. Different letters (a and b) over the bars denote significant differences (Tukey's test, $p<0.05$).

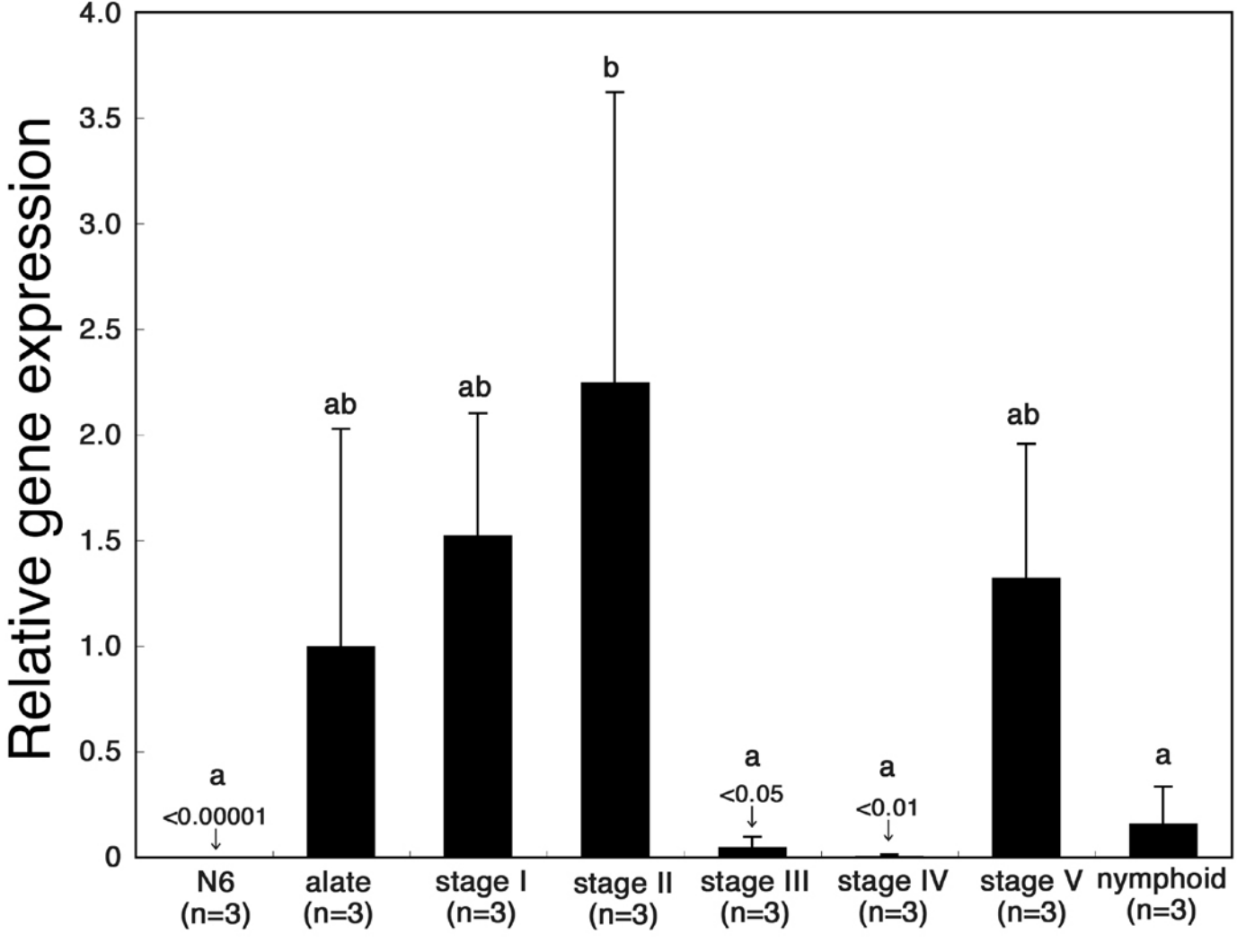
Fig. 4

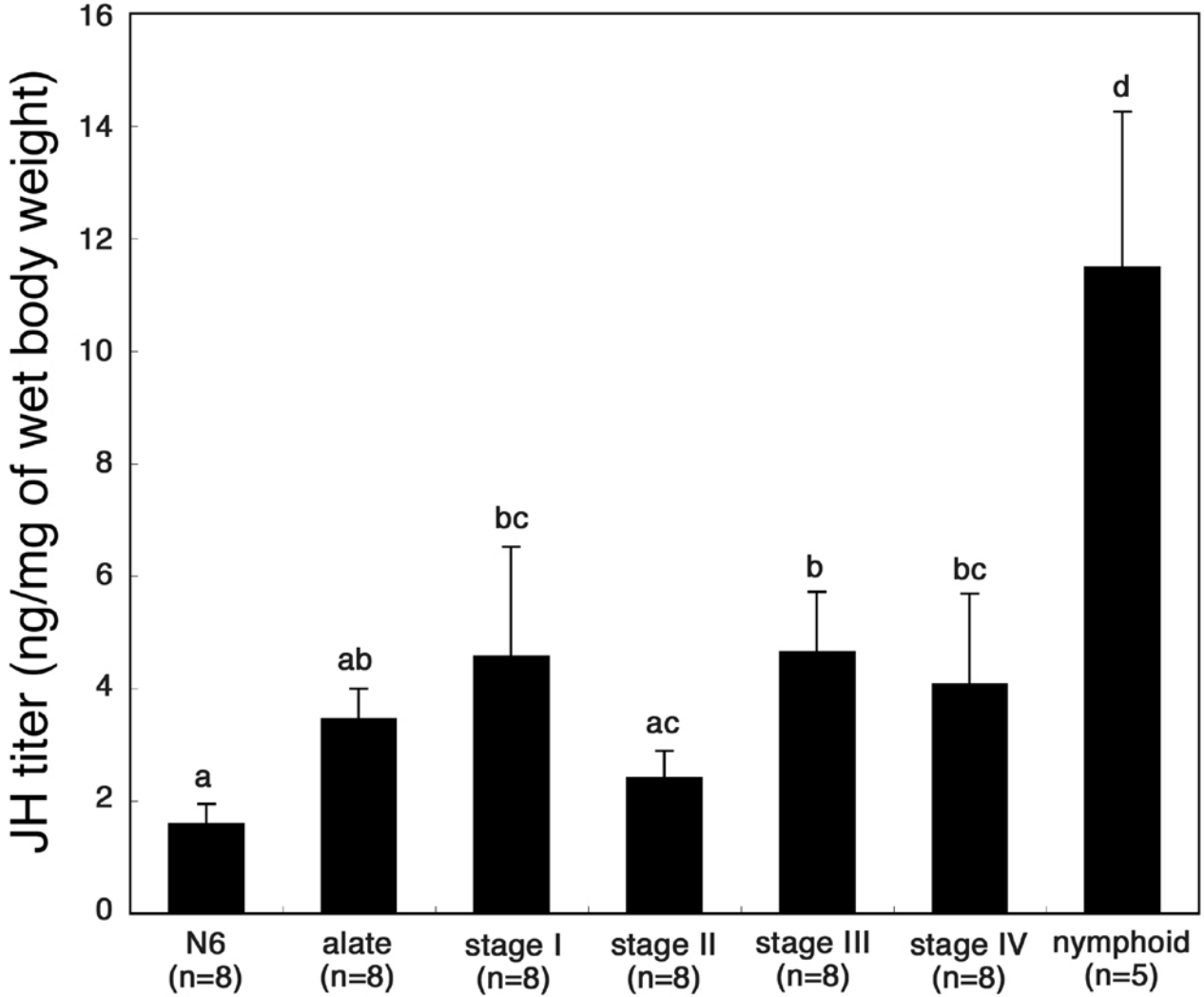
Juvenile Hormone III titres of individuals at each developmental stage (mean ± S.D. values). Each sample was pooled from 5 individuals and was replicated 8 times (= 40 individuals) for N6, alates, and primary reproductives. Five repricates (= 25 individuals) were analyzed for nymphoids. Different letters (a-d) over the bars denote significant differences (Tukey's test, $p<0.05$).


A
Number of colony members
110
100
90
80
70
60
50
40
30
20
10
0
a
b
c
c
d
stage I (n=45)
stage II (n=55)
stage III (n=55)
stage IV (n=56)
stage V (n=10)


B
Number of eggs per colony
55
50
45
40
35
30
25
20
15
10
5
0
a
b
c
d
e
stage I (n=45)
stage II (n=55)
stage III (n=55)
stage IV (n=56)
stage V (n=10)


A (N6)
E (stage III)
B (alate)
F (stage IV)
C (stage I)
G (stage V)
D (stage II)
H (nymphoid)


Relative gene expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
a
<0.00001
ab
ab
b
a
<0.05
a
<0.01
ab
a
N6 (n=3)
alate (n=3)
stage I (n=3)
stage II (n=3)
stage III (n=3)
stage IV (n=3)
stage V (n=3)
nymphoid (n=3)


JH titer (ng/mg of wet body weight)
16
14
12
10
8
6
4
2
0
a
ab
bc
ac
b
bc
d
N6 (n=8)
alate (n=8)
stage I (n=8)
stage II (n=8)
stage III (n=8)
stage IV (n=8)
nymphoid (n=5)