Ovarian development and insulin-signaling pathways during reproductive differentiation in the queenless ponerine ant *Diacamma* sp.

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ABSTRACT

In many social hymenopteran species, workers possess functional ovaries that are physiologically inactive in the presence of queens. We investigated the ovarian regulatory mechanism of workers and reproductives in a queenless ponerine ant, *Diacamma*, using histological and molecular techniques. In this ant, clear reproductive differentiation occurs via a highly sophisticated dominance behavioral interaction called “gemmae mutilation.” This clear and rapid bifurcation of reproductive physiology allows us to elucidate the detailed ovarian differentiation process. Histological characteristics of functional ovaries (fusomes and ring canals) were found in both workers and reproductives, suggesting that early oogenesis is not blocked in workers. Since insulin/insulin-like growth factor signaling (IIS) is known to control insect reproduction, orthologs of 2 positive IIS regulators, insulin receptor and serine-threonine kinase Akt (protein kinase B), were cloned in *Diacamma* (*DiaInR*, *DiaAkt*); their expression

patterns during reproductive differentiation were examined by real-time quantitative polymerase chain reaction; *DiaInR* and *DiaAkt* were strongly expressed in the gasters of reproductives. Whole-mount *in situ* hybridization of ovaries indicated that *DiaInR* and *DiaAkt* were expressed in nurse cells, oocytes, and upper germarial regions of reproductives but not of workers. Our data suggest that the IIS pathway accounts for reproductive differentiation in late oogenesis.

Key words: reproductive division of labor, insulin signaling, ovary, ponerine ant

1. Introduction

Reproductive division of labor is a fundamental characteristic of social insects. In social Hymenoptera, reproductive differentiation is generally controlled at 2 levels: ontogenic differentiation of ovariole number during larval and pupal development (Hölldobler and Wilson, 1990; Schmidt-Capella and Hartfelder, 1998, 2002; Boleli et al., 1999) and physiological differentiation of ovarian activity after becoming adults (West-Eberhard, 1979; Gadagkar, 1990; Turillazzi and West-Eberhard, 1996). The former is observed in highly eusocial species having morphological castes, whereas the latter occurs in almost all eusocial hymenopterans, because workers generally possess ovaries that are kept inactive in the presence of the queen(s) (Bourke, 1988; Choe, 1988; Hölldobler and Wilson, 1990). In primitively eusocial wasps and queenless ponerine ants, all adult females in a colony have the same morphology and reproductive potential. Therefore, physiological ovarian differentiation is one of the fundamental mechanisms

underlying eusociality, and the suppression of ovarian activity, from an evolutionary point of view, may represent a primitive phase of worker sterility. Among several physiological factors, recent studies indicate that the insulin/insulin-like growth factor signaling (IIS) pathway plays important roles in caste differentiation (Wheeler et al., 2006; de Azevedo and Hartfelder, 2008). This pathway has pleiotropic effects on reproduction, growth, metabolism, and cell division (Oldham and Hafen, 2003; Wu and Brown, 2006; Mirth and Riddiford, 2007), which means that IIS may be involved at multiple levels in caste differentiation. However, IIS involvement has only been known in morphological caste differentiation in honeybees (Wheeler et al., 2006; de Azevedo and Hartfelder, 2008). Considering the roles of IIS in caste differentiation and its regulatory functions in the ovaries of solitary insects (Riehle and Brown, 1999, 2002; Wu and Brown, 2006; Roy et al., 2007; Brown et al., 2008), we hypothesized that the IIS pathway was involved in the control of ovarian activity in social insects as well. In order to

understand adult ovarian differentiation, we carried out histological examinations on ovarian changes and molecular analyses of IIS dynamics during reproductive differentiation in the queenless ponerine ant *Diacamma* sp.

In most primitively eusocial wasps and queenless ponerine ants, unpredictable turnover of dominant egg layers makes it difficult to analyze the process of ovarian differentiation. In the queenless ponerine ant genus *Diacamma,* prospective egg layers can be detected, because reproductive differentiation is triggered by highly ritualized dominance behavior that is easily inducible in the laboratory. In this genus, all eclosed females have the potential to become reproductives, but only a single female possessing "gemmae," i.e., vestigial wings whose presence determines reproductives (Fukumoto et al., 1989; Peeters and Higashi, 1989; Gotoh et. al., 2005), becomes a dominant egg layer called a “gamergate” (functional queen in queenless ants; Peeters and Crewe, 1985; Peeters, 1991). The rest of the females

whose gemmae were mutilated by the gamergate become subordinate sterile workers (Fig. 1). Mutilated females lose their ability to mate (Allard et al., 2005), and they can reproduce only when the gamergate is absent (Fukumoto et al., 1989; Peeters and Higashi, 1989). This unique behavioral determination of reproductives in *Diacamma* enables us to investigate the precise process of ovarian differentiation under the control of age and dominant status.

In active ovarioles of Hymenoptera, cells called “cystoblasts” divide into cystocytes that later differentiate into a single oocyte and sister nurse cells in the germarium, the upper region of the ovariole (Gutzeit et al., 1993; Büning, 1994; Heming, 2003). During cystocyte division, conspicuous actin cytoskeletal structures called “fusomes” and “ring canals” are formed between the cells of cystocytes (Gutzeit et al., 1993; Heming, 2003). Fusomes function as anchors to settle the dividing cystocytes into a follicle (i.e., a set of one oocyte and sister nurse cells). Then, fusomes become ring canals to transfer the protein

and mRNA synthesized in nurse cells into an oocyte (Cooley and Theurkauf, 1994). These cytoskeletal structures are detectable during follicle formation but finally disappear before vitellogenesis (Gutzeit et al., 1993; Tanaka and Hartfelder, 2004). To establish the histological basis of ovarian differentiation between reproductives and workers, this study first revealed these ovarian cytoskeletal structures.

In the reproductive females of Diptera, insulin receptor (InR) is expressed in nurse cells and oocytes for oogenesis and embryogenesis (Garofalo and Rosen, 1988; Helbling and Graf, 1998). In addition, IIS is known to stimulate the secretion of ecdysteroid in follicle cells, resulting in vitellogenesis (Riehle and Brown, 1999, 2002; Roy et al. 2007; Brown et al. 2008). A similar mechanism would be expected to occur in adult reproductive differentiation in social insects. InR (also defined as insulin-like growth factor receptor) and Akt (protein kinase B) are major positive regulators of IIS (Oldham and Hafen, 2003; Wu and Brown, 2006; Mirth and Riddiford, 2007) that are conserved among

insect species. To test the activity of IIS in reproductive differentiation, *InR* and *Akt* homologs were cloned in *Diacamma*, and their expression dynamics were monitored by real-time quantitative polymerase chain reaction (qPCR). Furthermore, whole-mount *in situ* hybridization was performed in ovaries to confirm the detailed localization and differential expressions of these homologs in the ovaries of workers and reproductives.

2. Materials and methods

2.1. Insects

Complete colonies of *Diacamma* sp. (the only species of *Diacamma* in Japan) were excavated in Nakijin, Kenmin-no mori (Onna), Hantagawa Park (Naha) and Sueyoshi Park (Naha), in Okinawa, Japan. Gamergate-right colonies containing 50–200 workers were reared in plastic artificial nests (dimension: 7.7 × 10.8 × 3.2 cm) filled with moistened plaster. The artificial nests were placed in a plastic arena. To create colonies that lack gamergates (orphan colonies), 12–15 mutilated workers were isolated and kept in small artificial nests (dimension: 4.8 × 6.2 × 2.5 cm) as described above. They were maintained at 23°C under a light-dark (LD) 12:12 h photoperiod and fed with chopped mealworms (*Tenebrio molitor* larvae) 3 times a week. Honey water was supplied *ad libitum*.

2.2. Induction of reproductives and sterile workers

A newly eclosed female who has had no interaction with nestmates is defined as an “intact callow” (IC); ICs can differentiate into either egg layers or sterile workers depending on the colony status. In the present study, an IC was obtained by isolating a female pupa. The pupal cocoon was removed to help her eclosion. In an orphan colony, a newly eclosed female retains gemmae on the thorax and becomes a prospective egg layer (Fig. 1, Fukumoto et al., 1989; Peters and Higashi, 1989). Such an unmated prospective egg layer is referred to as a future gamergate (FG). The induced FGs began laying eggs within 14 days after eclosion (see Results). To obtain workers, newly mutilated females (i.e., new workers) were collected from gamergate-right colonies. Usually, the gemmae of eclosed callows are mutilated, mainly by gamergates, within a few hours (Okada, personal Observation; Fukumoto et al., 1989; Peeters and Higashi, 1989). Workers of the following 5 age categories were collected from gamergate-right colonies: day-1, day-7, day-14, day-90, and day-180. The day-90 category included workers aged between 15 and 90

days. The day-180 category included workers older than 180 days. FGs of 3 age categories were induced: day-1, day-7, and day-14. Gamergates older than 180 days were collected from stock colonies.

In orphan colonies, a few highly ranked mutilated females (dominant workers) can lay male-destined haploid eggs, whereas the rest of the females are kept sterile (subordinate workers) (Peeters and Tsuji, 1993). We obtained the dominant and subordinate workers by creating orphan colonies consisting of 12–15 mutilated workers. These orphan colonies were kept at least 2 weeks, after which, all the workers were dissected to examine their ovarian development. Workers with fully developed ovaries were designated as dominant workers, whereas those with undeveloped ovaries were regarded as subordinate workers (Peeters and Tsuji, 1993).

2.3. Ovary dissection and histology

Ovaries were dissected in 1× PBS buffer. Then, the ovariole sheath was

carefully removed with sharp forceps. Individual ovarioles were fixed in 4% paraformaldehyde in 1 × PBS for 1 h, then rinsed in 1 × PBT (0.1 % Tween 20 in PBS) for 1 h. Subsequently, these samples were incubated for 1 h in staining reagent containing 0.67 U/ml of rhodamine-phalloidin (Molecular Probes, OR, USA) and 1 µg/ml of Hoechst 33342 trihydrochloride hydrate (Molecular Probes) diluted in 1 × PBT. Rhodamine-phalloidin stains actin cytoskeleton, such as fusomes and ring canals, whereas Hoechst visualizes nuclei containing DNA. Stained ovarioles were repeatedly rinsed in PBS for 1 h and then mounted on glass slides with VECTASHILD mounting media (Vector, Burlingame, CA, USA). The whole-mount ovary preparations were analyzed with a fluorescence microscope (BX-51; Olympus, Tokyo, Japan) and a laser confocal microscope (LSM-DUO; Zeiss, Oberkochen, Germany). All staining procedures were performed at room temperature.

2.4. Measurement and staging of oocytes

Adult females in *Diacamma* sp. generally have 12 ovarioles per individual (6 ovarioles per ovary). Of these, the most developed ovariole was used to evaluate the ovarian activity of the female individual under investigation (Tsuji et al., 1999). Oocyte length was measured on the basis of captured digital images (DP-50 CCD; Olympus) using the image-analysis software Image J (National Institutes of Health). Oocyte stage was determined by King's criteria (King, 1970; Table 1), which describes each stage as the size ratio of an oocyte against a sister nurse cell cluster (nurse chamber). As an index of oocyte number per ovariole, the number of oocytes exceeding stage 8 (i.e., the stage at which an oocyte is distinguishable from nurse cell in size; Table 1) was counted.

2.5. Cloning and quantification of insulin-signaling genes

Total RNA from the gasters of 6–8 ants was extracted by the SV total RNA isolation system (Promega, Madison, WI, USA), and 800 ng of total

RNA was reverse transcribed using Oligo-dT(20) Primer (Invitrogen, Carlsbad, CA, USA) and Super Script III reverse transcriptase (Invitrogen). Using the reverse-transcribed cDNAs as templates, gene fragments of *InR*, *Akt*, and *actin* were obtained by PCR amplification with the specific degenerate primers (Table 2) and Ex Taq polymerase mix (Takara, Otsu, Japan). PCR conditions were 94°C for 30 s, 45–60.8°C for 30 s, and 72°C for 3 min for 35 cycles. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using SYBR green (Applied Biosystems, Foster City, CA, USA) with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). For relative quantification of the targeted transcripts, cycle threshold (Ct) values were calculated by the relative standard curve method and normalized to the *actin* gene. Levels of the targeted gene expressions were determined as expressions relative to IC. Reactions were technically triplicated to ensure accuracy of amplification. Primer sequences for qRT-PCR (Table 2) were designed by the Primer Express

ver. 2.0.0 software (Applied Biosystems).

2.6. In situ *hybridization*

Since the histological difference became obvious at day 7 (see Results), *in situ* hybridization was performed on the ovarioles of day-7 workers and FGs. Dissected ovarioles were transferred into a 1:4 mixture of 4% paraformaldehyde in PBS and heptane for 20 min and rinsed in PBT (0.1 % Tween 20 in PBS) 5 times. These ovarioles were digested with proteinase K (4 µg/ml) for 15 min and then rinsed 2 times in PBT. After postfixation in 4% paraformaldehyde for 20 min, the ovarioles were rinsed 5 times in PBT and once in a 1:1 mixture of hybridization buffer (HB) and PBST and then transferred to HB at 65°C for at least 2 h. The prehybridization buffer was replaced with RNA probe in HB (5 ng/µL) and incubated overnight at 60°C. The digoxigenin (DIG)-labeled RNA probe was prepared from the cloned partial sequences following the procedures of Osborn and Dearden

(2005). Tissue was then rinsed in HB (2:1), 1:1 mixtures of HB and PBST, and washed in PBST 5 times. The DIG hapten was detected with a 1:500 dilution of anti-DIG-alkaline phosphatase antibodies (Roche Applied Science) in PBST for 1 h at room temperature. The tissue was washed 5 times in PBST and 2 times in the developing solution [0.1M Nacl, 0.1 M Tris-HCl (pH 9.5), 0.05 M $MgCl_2$, 0.1% Tween 20]. The alkaline phosphatase enzyme was detected with NBT/BCIP stock solution (Roche Applied Science). Tissues were destained in methanol and rehydrated in PBST.

3. Results

3.1. *Early oogenesis: Fusome and ring canal formation*

Fusomes and ring canals, which are known to be characteristics of cystocyte division (Cooley and Theurkauf, 1994; Heming, 2003), were observed in ovaries of ICs (Fig. 2a), suggesting that emerging callows had already begun cystocyte division. These structures were observed in FGs and workers at day 7 (Figs. 2b, c, d, e). Thus, cystocyte division continued even after gemmae mutilation. Fusomes were accurately transformed into ring canals in the upper germarium of day-7 workers, where the signs of programmed cell death (apoptosis) had been observed in honeybee workers (Tanaka and Hartfelder, 2004). Fusomes and ring canals were continuously observed in day-14 and day-90 workers and in all stages of reproductive females (data not shown).

3.2. *Late oogenesis: Oocyte growth and nurse cell degeneration*

The size of oocytes in FGs rapidly increased at day 7 and reached maximum size by day 14 (Fig. 3a). The number of oocytes increased as they matured (Figs. 3b, 3c). In contrast, oocyte size in workers did not increase for at least 14 days after eclosion (Figs. 3, 4). Although there was a slight increase in workers' oocyte size between day 14 and day 90 (P = 0.01, Student's t-test), their oocyte size never exceeded the size of the sister nurse chamber (Figs. 3, 4g). Specifically, young workers (day-90) had small oocytes in vitellaria, but they were arrested at stage 9 (Table 1).

Large nurse cell nuclei were observed in the developing follicles of FGs and in those of workers younger than 90 days. The densely stained small nurse cell nuclei were observed in the mature follicles of day-14 FGs and gamergates (Fig. 4d, arrowheads). These small nurse cell nuclei are characteristic of degenerating nurse cells that have finished providing materials to the oocyte (King, 1970). The degenerating nurse cells from several follicles, together with the

remnants of follicle cells, accumulate and eventually form a large yellow body (Fig. 4f, arrowhead) (Billen, 1985; Peeters, 1993; Büning, 1994). Interestingly, in some of the workers older than 180 days, similar densely stained small nurse cell nuclei, indicating cellular degeneration, were observed (4/17 workers; Fig. 4h, arrowheads). These old workers did not lay eggs, and the oocytes of these old workers were immature. However, the sister nurse cells of oocytes had begun to degenerate, indicating that inactive nurse cells of old workers lose their activity. In other old workers, oocytes and nurse cells were not detected (13/17). These findings suggest that degeneration of worker nurse cells and oocytes occurred only in old individuals.

3.3. *Insulin-signaling gene expression correlated with reproductive roles*

We successfully cloned partial sequences of orthologous genes for InR and Akt and termed them as *DiaInR* and *DiaAkt,* respectively

(Table 2). The cDNA fragment sizes were 410 bp (*DiaInR*) and 389 bp (*DiaAkt*). The sequence data was deposited in the DDBJ/EMBL/GenBank database under accession numbers: AB510470, AB510471.

Real-time qRT-PCR revealed that the expression level of *DiaInR* in the gaster was significantly higher in reproductives (FG, gamergates, or dominant workers) than in sterile counterparts of the same age (Fig. 5a; day-1: $P < 0.001$; day-7: $P = 0.001$; day-14: $P < 0.001$, day-180: $P = 0.006$; dominants vs. subordinates: $P = 0.004$, Student's *t*-test). The expression level of *DiaAkt* in the gaster also showed a clear difference between egg layers and sterile individuals (Fig. 5b; day-14: $P < 0.001$; day-180: $P < 0.001$; dominants vs. subordinates: $P = 0.002$, Student's *t*-test), but this difference was not detected in the early stages of differentiation (day-1: $P = 0.48$; day-7: $P = 0.20$, Student's *t*-test).

To test the differential expression of *DiaInR* and *DiaAkt* in the

ovary, whole-mount *in situ* hybridization was performed on the ovarioles of day-7 FGs and day-7 workers. *DiaInR* and *DiaAkt* mRNA were strongly expressed in the enlarged nurse cell cytoplasm (Fig. 6). *DiaInR* and *DiaAkt* were also detected in the developed oocytes and upper germarial regions of day-7 FGs (Fig. 6). The oocytes of day-7 FGs appeared to contain *DiaInR* and *DiaAkt* transcripts. In contrast, *DiaInR* and *DiaAkt* transcripts were not detected in day-7 workers.

4. Discussion

4.1. *Oogenesis in* Diacamma

Histological observations revealed that signs of early oogenesis, such as fusomes and ring canals, were clearly observed in the upper germarial region in both reproductives and mutilated females (Fig. 2). In honeybee worker ovarioles, disorganization of the actin cytoskeleton of fusomes and ring canals has been observed, indicating that their sterility is controlled not only during oocyte growth but also at oocyte formation (Tanaka and Hartfelder, 2004). In *Diacamma*, oocyte formation is not impaired even after gemmae mutilation in young workers, suggesting that the control of oogenesis is not accompanied by a fundamental change in early oogenesis. In old workers (day-180), fusomes and ring canals were not detected, and degeneration of nurse cells and oocytes occurred. Such an age-dependent decline in reproductive activity has been identified in several species of social hymenoptera (Jeanne, 1972; West-Eberhard, 1978, 1981; Yamane,

1986; Higashi et al. 1994; Monnin and Peeters, 1999). Degeneration of nurse cells and oocytes may reduce fertility in old workers in *Diacamma* and other social hymenopterans. Such predominant sterility in old females might facilitate the division of labor, leading to a colony-level benefit in social organization. The ovaries of these workers seem to follow a certain chronological schedule that may have derived from ancestral hormonal and/or physiological constraints related to reproduction (the “ovarian ground plan” defined by West-Eberhard, 1996). Although the mechanism of degeneration/re-absorption of nurse cells and oocytes is presently unclear, unnecessary old follicles collapse when they reach a certain age (90–180 days).

4.2. *Insulin signaling and reproductive division of labor*

The expression levels of the insulin receptor (*DiaInR*) and protein kinase B (*DiaAkt*) correlated with the reproductive activity observed in gasters. The insulin receptor and downstream IIS factors are

up-regulated in various insect tissues to regulate metabolism, reproduction, and cellular growth and proliferation (Wu and Brown, 2006). Besides ovarian activity itself, substantial nutritional uptake and protein synthesis are required for reproduction (Wheeler, 1996) so that the IIS may alter metabolic activity in the gut and fat bodies in the gasters. High levels of *DiaInR* and *DiaAkt* in the gasters and ovaries of reproductives may enable them to translate a nutritional signal into oogenesis. *In situ* hybridization revealed that *DiaInR* and *DiaAkt* were strongly expressed in the nurse cells of reproductives, supporting the idea that the IIS mediates reproductive differentiation via the activity of nurse cells. The localizations of IIS genes in nurse cells and oocytes closely resembled those in fruit flies and mosquitoes (Garofalo and Rosen, 1988; Helbling and Graf, 1998; Riehle and Brown, 2002). Considering the dipteran studies (Garofalo and Rosen, 1988; Fernandez et al., 1995; Ruan et al., 1995), transcribed *DiaInR* and *DiaAkt* in nurse cells and oocytes may enhance the synthesis of materials that function

in oogenesis and future embryogenesis (e.g., *vasa*, *nanos*; Khila and Abouheif, 2008; Tanaka and Hartfelder, 2009). The expression levels of *DiaInR* and *DiaAkt* in nurse cells and oocytes became stronger in larger follicles. Interestingly, in honeybee and stingless bee ovarioles, the germline marker gene *vasa*, which is necessary for oocyte maturation (Gavis et al., 1996), was strongly expressed in larger follicles in a very similar pattern to *DiaInR* and *DiaPKB* expression (Tanaka and Hartfelder, 2009), implying that expressions of these molecules are somehow correlated. *DiaInR* and *DiaAkt* were also detected in the upper germarial region of day-7 FGs. Since the IIS promotes cellular proliferation in general, IIS may accelerate cystocyte division in the upper germarium.

Our histological and molecular data suggest that reproductive differentiation in *Diacamma* is not controlled at early oogenesis but by the activity of IIS in nurse cells and oocytes at late oogenesis. The IIS pathway that mediates nutritional conditions and ovarian activity in

solitary species (Riehle and Brown, 1999, 2002; Brown et al., 2008) is therefore suggested to be co-opted in the dominance-dependent reproductive differentiation in *Diacamma*. Since mutilated females can still lay haploid eggs in the absence of a gamergate (Fukumoto et al., 1989; Peeters and Tsuji, 1993) or in large colonies where gamergate policing is not sufficient (Nakata and Tsuji, 1996; Kikuchi et al., 2008), it is reasonable to assume that the arrest of IIS activity at late oogenesis causes retention of egg-laying ability. Such worker reproductive potential and its social suppression are shared characteristics of most hymenopteran species in which workers still retain reproductive ability. Therefore, the systems observed in *Diacamma* may exist in other social hymenopterans. The dynamics of insulin-like peptides and interactions between IIS and other reproductive regulators such as juvenile hormones and ecdysteroids (Röseler et al., 1984, 1985; Robinson et al., 1992; Bloch et al., 1996, 2000; Robinson and Vargo, 1997; Brent et al., 2003, 2006; Sommer et

al., 1993) are fundamental foci for further understanding of reproductive division of labor. Through comparative analyses of ovarian histology and gene regulatory networks for oogenesis (e.g., Khila and Abouheif, 2008; Tanaka and Hartfelder, 2009), the links between social interaction and the arrest of oogenesis can provide developmental and evolutionary insights into the reproductive division of labor.

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Figure Captions

Figure 1. Schematic diagram of the reproductive differentiation in *Diacamma* sp. Gemma-mutilated females differentiate into workers whereas females that retain gemmae become future gamergates and with developed ovaries.

Figure 2. Fusome and ring canal formation in callows, future gamergates (FGs) and workers. (a) Ovariole of intact callow (IC). Previtellogenic oocytes (black arrowheads) and polyfusomes (white arrowheads) were observed. (b, d) Apical region of germarium of day-7 workers and day-7 FGs. Fusomes (arrowheads) were clearly observed in both FGs and mutilated workers. The regions where ring canals were formed (boxes) were magnified in c and e. (c, e) Ring canals of day-7 workers (c) and day-7 FGs (e). (a, b, d): Fluorescent microscopy, rhodamine-phalloidin stained. (c, e): Confocal microscopy,

rhodamine-phalloidin stained. Bars indicate 100 µm (b,d) and 10 µm (c, e).

Figure 3. Transition of oogenesis during the differentiation into future gamergates and workers: (a) largest oocyte length, (b) number of oocytes over stage 8, and (c) stages of largest oocytes. Black dots: future gamergates (FGs) or gamergates; white dots: mutilated workers (Ws); grey dots: intact callows (IC). The numbers of samples are as follows: 9 (IC), 5 (day-1 Ws), 7 (day-7 Ws), 6 (day-14 Ws), 12 (day-90 Ws), 17 (day-180 Ws), 11 (day-1 FG), 11 (day-7 FG), 6 (day-14 FG), 3 (day-90 FG), and 3 (day-180 gamergate).

Figure 4. Fluorescent microscopy of Hoechst-stained *Diacamma* ovarioles. (a) Intact callow (IC), (b) day-7 worker, (c) day-14 worker, (d) day-90 worker, (e) day-180 workers, (f) day-7 future gamergates, (g) day-14 future gamergates, (h) gamergates. Arrowheads in (d) and

(h) indicate degenerating nurse cell nuclei. An arrowhead in (f) shows yellow body. Bars indicate 100 µm.

Figure 5. The expression levels of genes in insulin signaling in the gaster of *Diacamma*. (a) *Insulin receptor* homolog (*DiaInR*) and (b) *Akt* homolog (*DiaAkt*) in *Diacamma*. Grey bars: intact callow (IC). White bars: sterile workers. Black: reproductives; W: worker; FG: future gamergate; sub: subordinate worker in orphan colony; dom: dominant egg layer in orphan colony; G: gamergate. Mean ± SD, n = 3. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, Student's *t*-test.

Figure 6. Patterns of *DiaInR* and *DiaAkt* expressions in the ovaries of *Diacamma*. (a, e) *DiaInR and DiaAkt* mRNAs were localized in nurse cell cytoplasm (black arrowheads), upper germarial region (black arrows), and oocyte (white arrowheads) of day-7 future gamergate. (b, c, d) No signal of *DiaInR and DiaAkt* was detected in day-7 worker and controls

with sense probes. The pattern of *DiaAkt* expression almost overlapped with the *DiaInR* pattern. Bars indicate 100 µm.

Tables

Table 1. Developmental stages defined by King (1970), on the basis of ovarian development in *Drosophila*.

Table 2. Primer sets used for the gene cloning and qRT-PCR examinations of insulin signaling genes (InR and Akt/PKB) and for the actin gene (endogenous control for qRT-PCR).

Fig. 1.

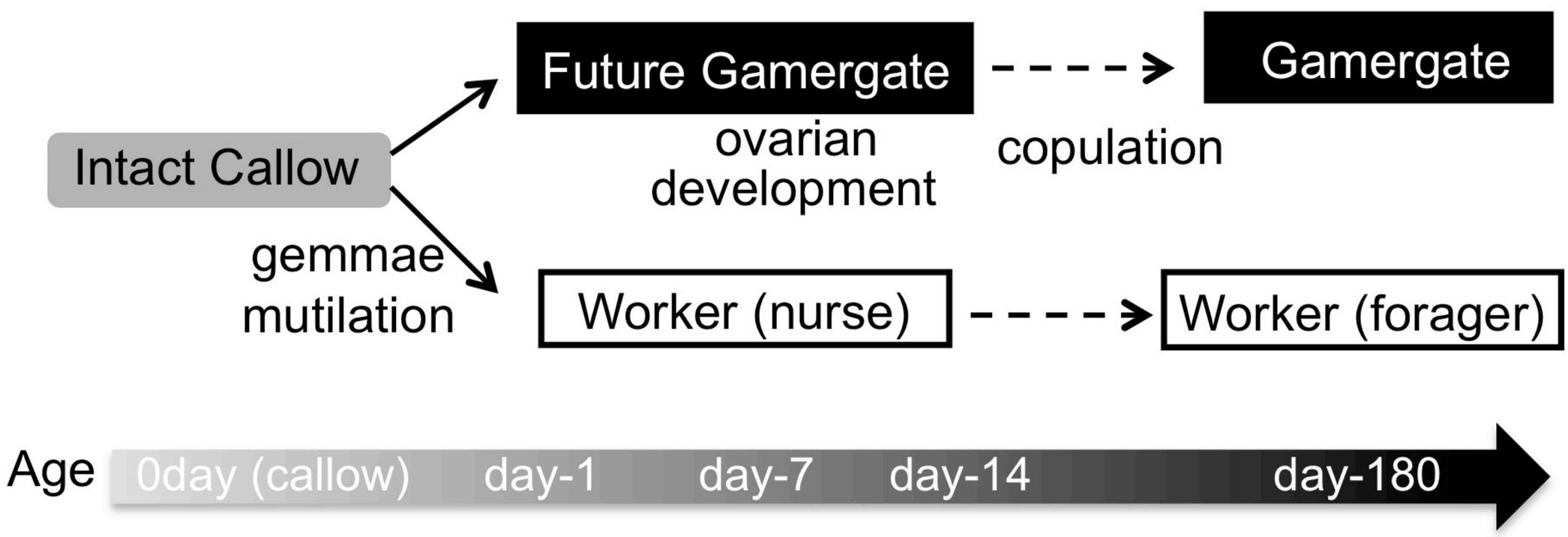
Future Gamergate
Gamergate
ovarian development
copulation
Intact Callow
gemmae mutilation
Worker (nurse)
Worker (forager)
Age
0day (callow)
day-1
day-7
day-14
day-180

Fig. 2.

Fig. 3.

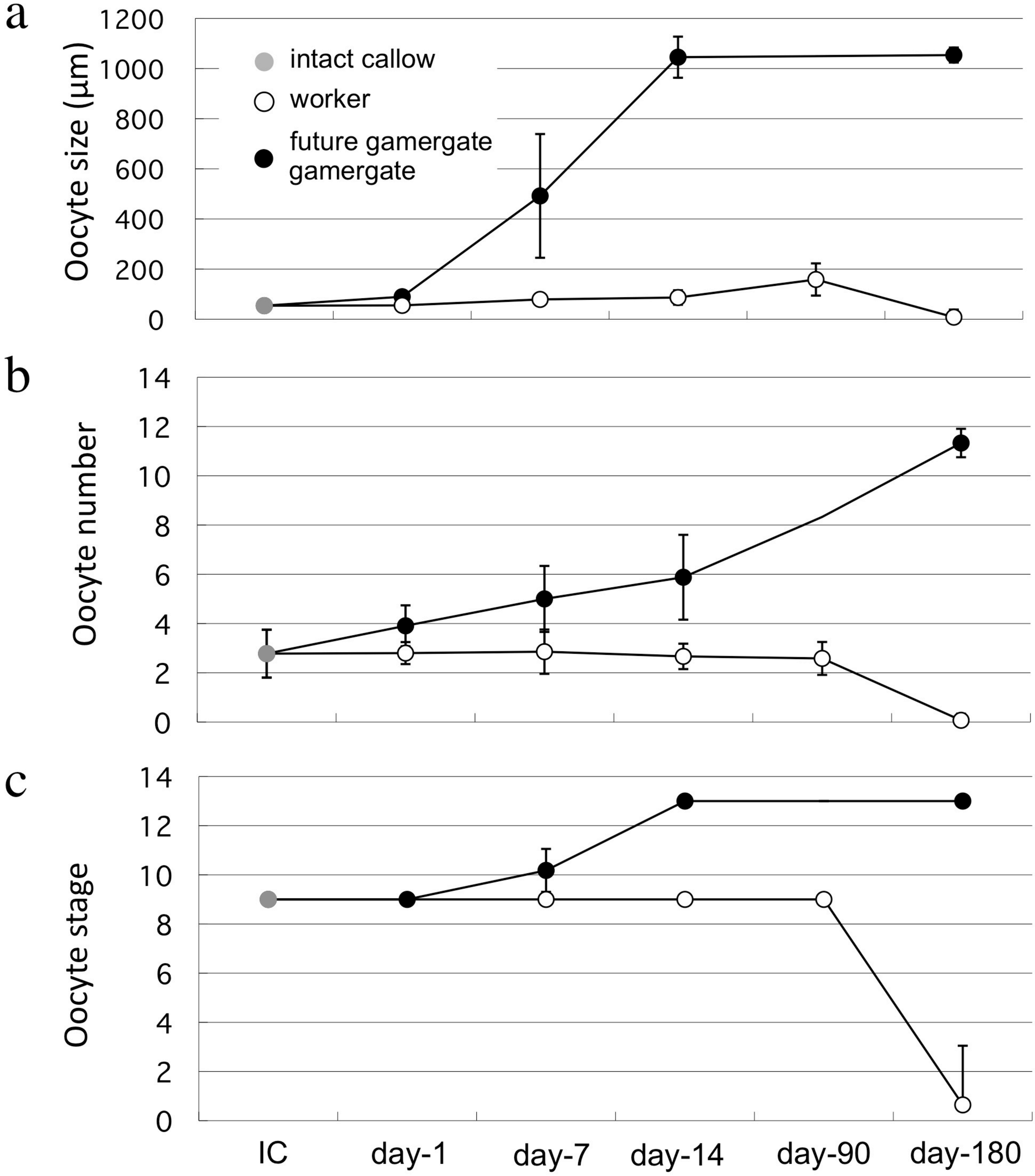
a
Oocyte size (μm)
1200
1000
800
600
400
200
0
intact callow
worker
future gamergate
gamergate
b
Oocyte number
14
12
10
8
6
4
2
0
c
Oocyte stage
14
12
10
8
6
4
2
0
IC
day-1
day-7
day-14
day-90
day-180

Fig. 4.

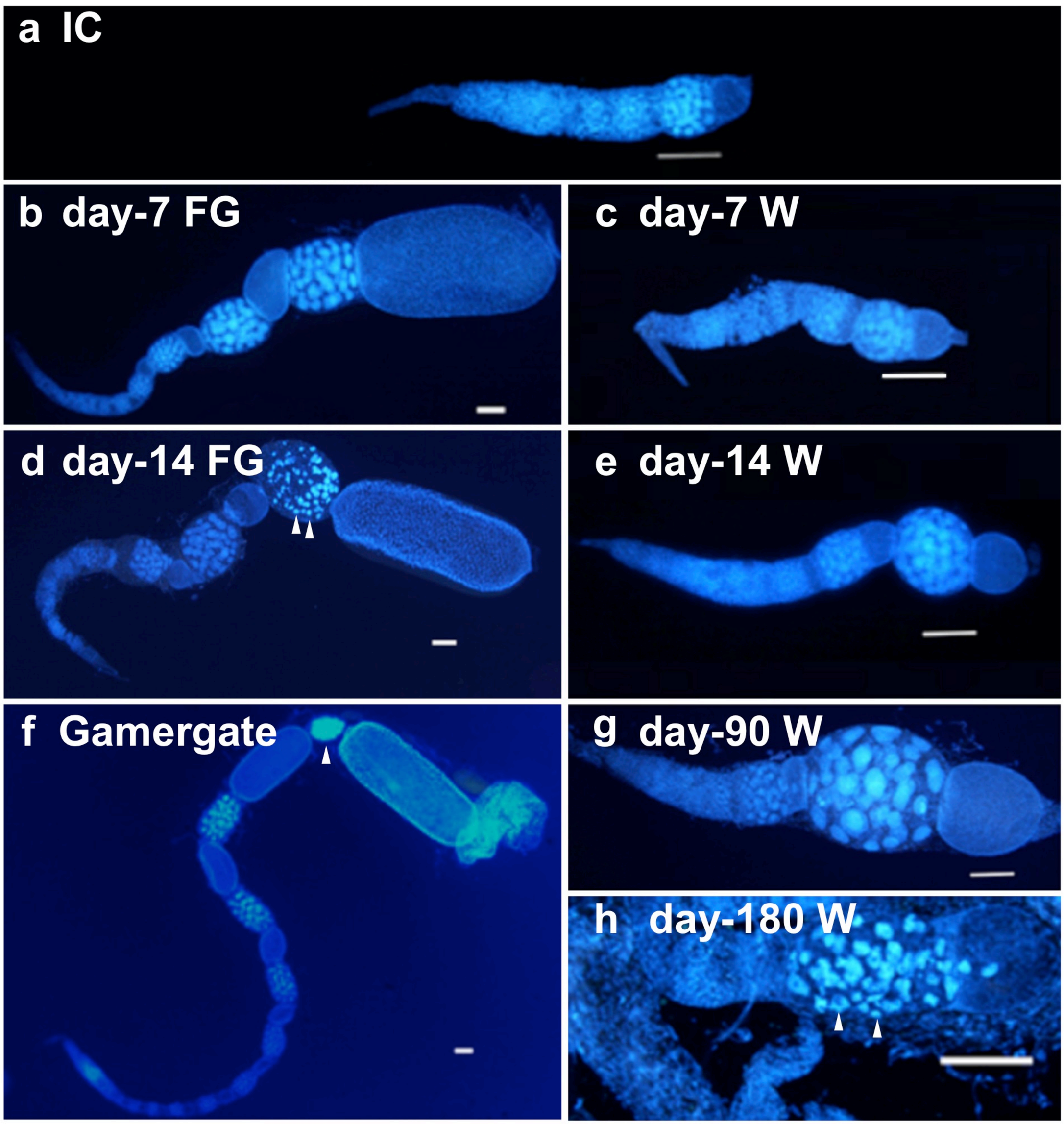
a IC
b day-7 FG
c day-7 W
d day-14 FG
e day-14 W
f Gamergate
g day-90 W
h day-180 W

Fig. 5.

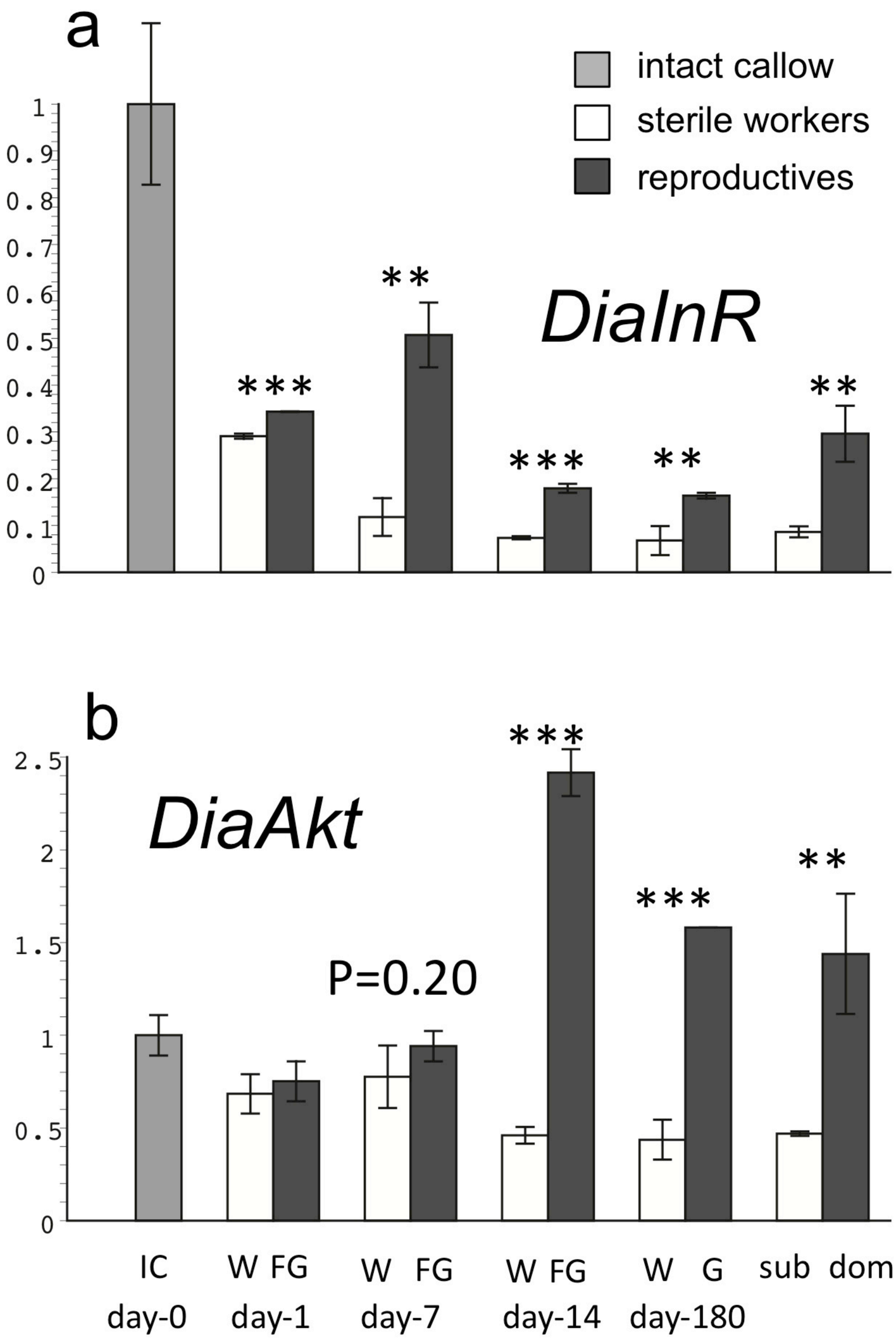
a
intact callow
sterile workers
reproductives
DiaInR
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
**
***
**
**
b
DiaAkt
P=0.20
***
**
2.5
2
1.5
1
0.5
0
IC
W FG
W FG
W FG
W G
sub dom
day-0
day-1
day-7
day-14
day-180

Fig. 6.

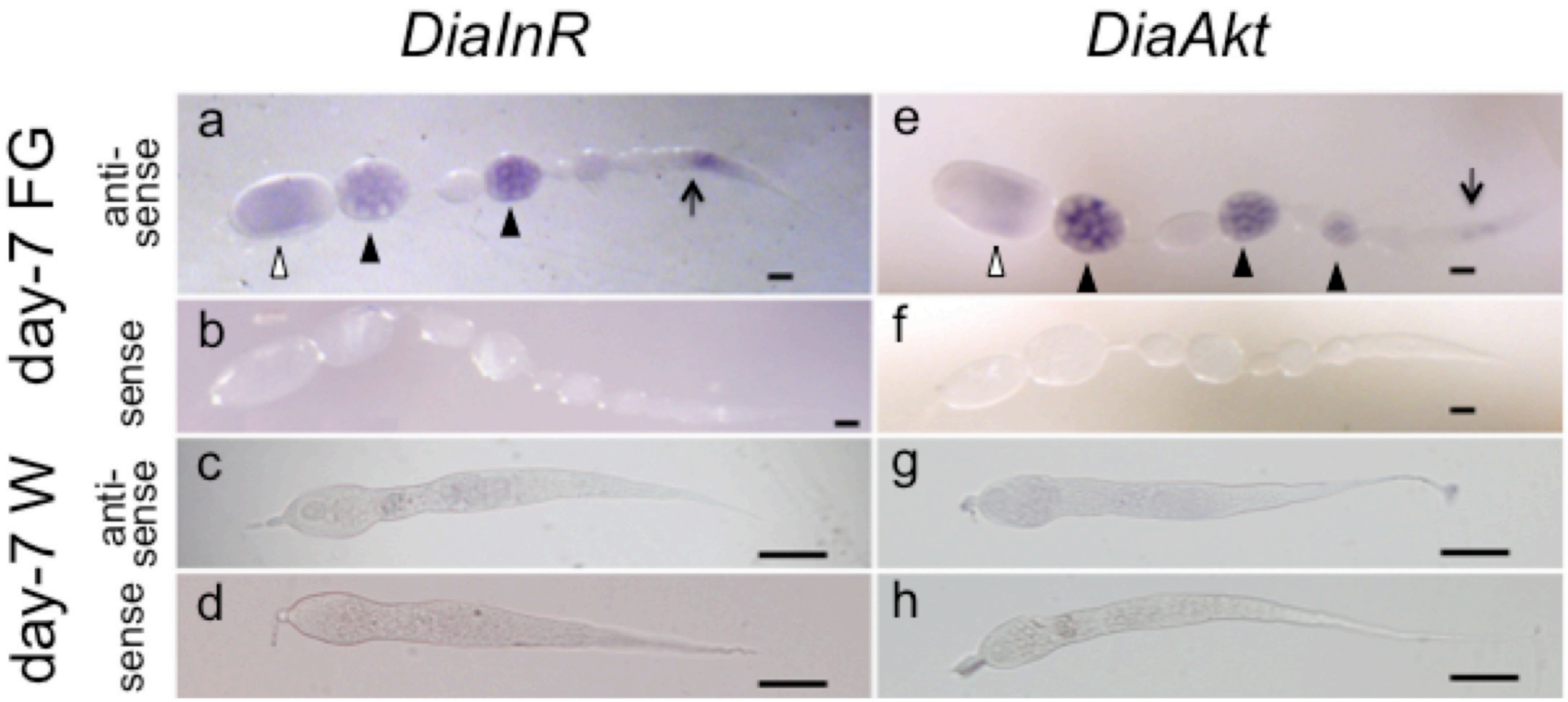
DiaInR
DiaAkt
day-7 FG
day-7 W
anti-sense
sense
anti-sense
sense
a
b
c
d
e
f
g
h

Table 1.

Stage	Description (Modified from King 1970)
St.7	Nurse cells and oocyte are equal in size.
St.8	The oocyte becomes significantly larger than average nurse cell.
St.9	The oocyte volume becomes 1/3 to equal of the nurse chamber.
St.10	The oocyte volume becomes equal to 3 times of the nurse chamber.
St.11	The oocyte volume becomes more than 3 times of the nurse chamber.
St.12	The oocyte has almost reached its maximum size. Nurse cell nuclei are stained densely.
St.13	Nurse cell nuclei start to degenerate.
St.14	Fully grown oocyte.

Table 2.

Genes	Degenerate primers	Realtime quantitative RT PCR primers	Fragment length	Similarity (blast x)
InR (insulin-like growth factor receptor)	F: 5'- CTGCGVMGDCCBTSCAACTA-3'	F: 5'- GTCAGCCTGGCGGTCAGT -3'	410bp	Similar to insulin receptor (*Nasonia vitripennis*, XP_001606180.1, 2e-39)
	R: 5'- CAAAYBATRAABGCVCTCATDATGAT -3'	R: 5'- CAACGCCATCGGCATCA -3'		Similar to Insulin-like growth factor receptor (*Apis melifera*, XP_001121628.1, 6e-34)
Akt (serine threonine kinase)	F: 5'- AARGACGAAGTNGCNCACAC-3'	F: 5'- AACCAAGGGCCGATATAATCTCT -3'	389bp	Similar to rac serine/threonine kinase (*Nasonia vitripennis*, XP_001605990, 1e-61)
	R: 5'- CCCCACCAATCNACAGCTCGTCC -3'	R: 5'- AGTGTTTGGCGAAGACCGTACT -3'		Similar to Akt1 isoform A (*Apis melifera*, XP_396874, 4e-61)
Actin	F: 5'- CYATYGGYAAYGARAGRTTCCGTTG -3'	F: 5'- GCTAAGGCAGTGATCTCCTTCTG -3'	229bp	beta-actin (*Menidia estor*, ACS45392, 6e-13)
	R: 5'- TATTTCYTYTCRGGTGGHRCGATGAT -3'	R: 5'- GGCGGCACCACGATGTA -3'		Actin (*Apis melifera*, XP_393368, 6e-13)