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Female-specific wing degeneration caused by ecdysteroid in the Tussock Moth, *Orgyia recens*: Hormonal and developmental regulation of sexual dimorphism

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Abstract

Females of the tussock moth *Orgyia recens* have vestigial wings, whereas the males have normal wings. During early pupal development, female wings degenerate drastically compared with those of males. To examine whether ecdysteroid is involved in this sex-specific wing development, we cultured pupal wings just after pupation with ecdysteroid (20-hydroxyecdysone, 20E). In the presence of 20E, the female wings degenerated to about one-fifth their original size. In contrast, the male wings cultured with 20E showed only peripheral degeneration just outside the bordering lacuna, as in other butterflies and moths. TUNEL analysis showed that apoptotic signals were induced by 20E over the entire region of female wings, but only in the peripheral region of male wings. Semi-thin sections of the wings cultured with ecdysteroid showed that phagocytotic hemocytes were observed abundantly throughout the female wings, but in only peripheral regions of male wings. These observations indicate that both apoptotic events and phagocytotic activation are triggered by ecdysteroid, in sex-specific and region-specific manners.

Keywords: wingless moth; ecdysone; sex hormone; TUNEL ; apoptosis; phagocytosis

Abbreviation:

BL	bordering lacuna
20E	20-hydroxyecdysone
PBS	phosphate-buffered saline
E	ecdysone
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
EcR	ecdysone receptor

Introduction

Dispersal polymorphisms in insects have played an important role in their evolutionary success. Insects from many orders develop winged dispersal morphs. Wingless morphs are of special interest to understand a fitness trade-off between flight capability and reproduction and the physiological basis of adaptation (Zera and Denno, 1997). Polymorphic development in insects has been studied at various levels: evolution, developmental, hormonal, genetic and environmental (Nijhout, 1999). Several studies suggested that polyphenic development is controlled by insect hormones. However, very little is known about the effects of hormones on target tissues in wing polyphenic species.

In butterflies and moths, the shape of the adult wing is defined by the position of a bordering lacuna (BL), which runs parallel to the periphery of pupal wings. The cells in the region distal to the BL degenerate and disappear during early pupal development (Dohrmann and Nijhout, 1988, Kodama *et al*., 1995), and the region proximal to the BL forms the adult wing. Our recent studies have demonstrated that this peripheral degeneration is induced directly by ecdysteroid (Fujiwara and Ogai, 2001). The peripheral degeneration of pupal wings is specific to Lepidoptera and is widely observed in most butterflies and moths, but we know several exceptional cases of "wingless" female moths. To explain the biological significance of this sexual dimorphism of wings of insects, it has been speculated that the nutritional resources used for flight may be reallocated to egg production in the female (Zera and Bottsford, 2001). There is a seasonal morph of a female *Orgyia thyellinam*; the summer-type has normal wings while the autumn-type exhibits shorter wings. In this insect, the normal-winged females lay smaller eggs than the short-winged females (Kimura and Masaki, 1977). Female-specific winglessness is observed widely among

several species of lepidopteran insects, Cossidae, Psychidae, Oecophoridae and some other groups (Common, 1970), suggesting that sex-limited winglessness is significant in adaptive change.

The developmental processes of two female wingless moths have been previously described. The tussock moth, *Orgyia leucostigma*, and the winter moth, *Nyssiodes lefuarius*, have normal wing discs during the larval stages in both sexes, but the female wings degenerate rapidly during early pupal stages (Nardi *et al*., 1991, Niitsu, 2001). However, the molecular and cellular mechanisms causing the female-specific winglessness are not yet fully understood. *Orgyia recens*, which lives in the cold regions of Japan, also displays female-specific winglessness (Yamada, 1982); we used the tussock moth to investigate the causes of sex-specific differentiation of wings. The developmental timing of female-specific wing degeneration in wingless tussock moths seems to parallel the increase in ecdysteroid titer in early pupal stages. This suggests that ecdysteroid induces massive cell death in the wings of female pupae, and results in substantial sex-specific winglessness. It has been reported that gender-specific differences of ecdysteroid titer in the tussock moth *Orgyia postica* may be correlated with their sexual dimorphism, although there is no direct evidence (Gu, *et al*., 1992). To test the effect of the hormone directly on the tissue, we isolated the wings from newly molted pupa of *O. recens* and cultured pupal wings with and without ecdysteroid. Futhermore, the ecdysteroid-inducible cell death regions were confirmed by TUNEL method on wings of both sexes. We also prepared semi-thin sections from cultured wings to observe ecdysteroid-inducible cell death *in vitro*.

Materials and Methods

Insects

Eggs of *O. recens* were collected at an apple orchard in Nagano, Japan. The larvae were reared on an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) developed for *Bombyx mori*. All developmental stages of *O. recens* were maintained under 16 h: 8 h photoperiod (light: dark) at 26 °C. Under these conditions, pupation occurs 2 days after the onset of wandering.

Tissue dissection and in vitro culture

In this study, we used only fore-wings. P-0 pupae (less than 30 min after pupation) were surface-sterilized in 70% ethyl alcohol for 30 sec and dissected on ice. The left forewings with cuticle were removed from the pupae by a scalpel as long as they were not sclerotized and rinsed three times in cold phosphate-buffered saline (PBS; 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4). Each wing was incubated at 25 °C on the surface of 1.0 ml Grace's medium (Gibco BRL, www.lifetech.com) including antibiotic-antimycotics (Gibco) in a 4-well multi dish (Nalge Nunc, www.nalgenunc.com). Ecdysone (E) (Sigma, www.sigmaaldrich.com) and 20-hydroxyecdysone (20E) (Sigma) were dissolved in 10% isopropanol, and added to the medium to give the desired concentration prior to use.

Microscopic observation

The excised left forewings with the pupal cuticle were rinsed in PBS and fixed immediately in formalin : 45% ethanol : acetic acid (2 : 6 : 1) overnight, and washed in three changes of PBS for 5 min. Wings were stained with either methyl green or Nuclear Fast Red for 30 min or 1 µg/ml DAPI (4'-6'-diamidino-2-phenylindole, Polysciences, Inc., www.polysciences.com) for 10 min. Excess dye was removed from the wings by three 5 min washes in water, and the wings were mounted in 50% glycerin. For semi-thin sections, dissected wings with the cuticle were immediately pre-fixed with 2.5% glutaraldehyde and 0.5% paraformaldehyde dissolved in PBS for 3 h. Tissues were rinsed twice with PBS and then post-fixed with PBS containing 2% OsO_4 for 3 h. Following three more rinses with PBS, wings were dehydrated with ethanol and acetone, and then embedded in Quetol 812 (Polysciences, Inc.). Semi-thin sections were cut with a Leica ultramicrotome (www.leica-microsystems.com) and stained with 0.1% toluidine blue in 1% aqueous borax solution. One-micron plastic sections were examined with a light microscope.

TUNEL analyses

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) analyses to visualize fragmented DNA were performed using ApoAlert® DNA Fragmentation Assay Kit (Clontech, www.clontech.com) for cultured wing discs and TACS™ *In Situ* Apoptosis Detection Kit (TACS Blue Label) (Trevigen, www.trevigen.com) for developing pupal wings.

The wings with the cuticle dissected from developing pupa were incubated for 60 min at room temperature with 50 µg/ml proteinase K solution. After washing with PBS for 15 min, the wings were treated with 2% hydrogen peroxide solution for 5 min, and incubated in 1x labeling buffer (100 mM Safe TdT buffer, 50 µg/ml bovine serum albumin (BSA), 60 µM beta-mercaptoethanesulfonic acid] for 5 min. The samples were incubated in TdT labeling buffer (15 units of TdT, 5 µM biotin-dUTP, 1 mM $CoCl_2$ in the 1x labeling buffer) and then rinsed with the TdT stop buffer (10 mM ethylendiaminetetraacetic acid (EDTA) in H_2O, pH 8.0) for 5 min, and washed with PBS for 2 min. The wings were incubated in 2 µg/ml streptavidin - horseradish peroxidase solution for 10 min, washed with PBS twice and incubated with TACS Blue label solution for 10 min, and examined by a light microscope.

The cultured wings were incubated in TdT labeling buffer (15 units of fluorescein-TdT 5 µM biotin-dUTP, 1 mM $CoCl_2$ in the 1x labeling buffer, then rinsed with the TdT stop buffer for 5 min, and examined by fluorescence microscopy with FITC filters.

Results

Sex-specific wing morphogenesis in developing pupa of O. recens

Males and females of *O. recens* cannot be discriminated in the larval stages. Even immediately after pupation, the wings of both sexes appear similar (Figs. 1A-e and 1A-f). The surface appearance of the external skeleton in both sexes does not seem to change during the pupal stages, although adult moths of *O. recens* show sexual dimorphism (Figs. 1A-a and 1A-b), especially in wing morphology (Figs. 1A-c and 1A-d). This observation indicates that the internal adult tissues of males and females develop differently during pupal stages. Male wings appear similar to typical Lepidopteran wings, covered with scales of normal size; female wings, on the other hand, decrease to about one-fifth their original

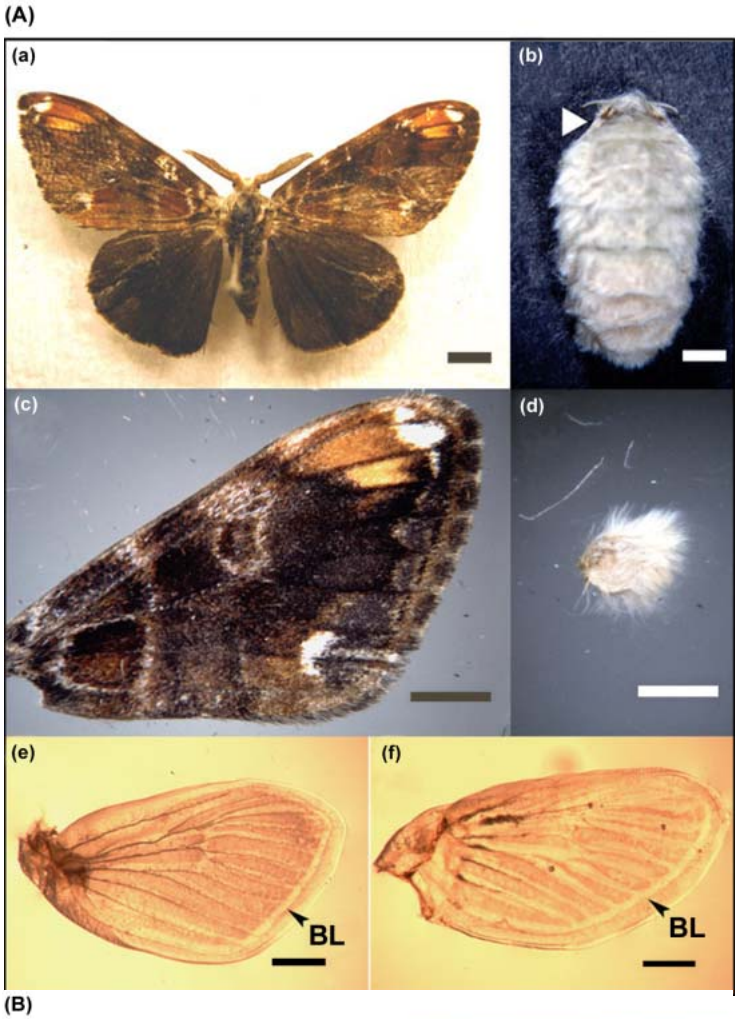

Figure 1. (Left) (A) (a and b): Adult *O. recens*. (a) Male whole view. (b) Female whole view. (c and d): Optical micrographs of the forewings of male (b) and female (c). (e and f): Dissected wings from pupae immediately after pupation (P-0) of male (e) and female (f). The bordering lacuna (BL) runs parallel to the periphery of pupal wings of both sexes. (B) Optical micrographs of the female pupal forewings at various stages, stained with methyl green. All wings were left in their cuticular pouch. (a and b): The pupal wings just after pupation (P-0). (b) Magnified view of the boxed area in (a). The BL (arrowhead) runs parallel to the wing edge. (c and d): Pupal wings at 1 day after pupation (P-1). No morphological change has occurred at this stage. (d) Magnified view of the boxed area in (c). (e and f): The pupal wings 36 h after pupation (P-1.5). Wing degeneration has occurred at this stage. (f) Magnified view of the boxed area in (e). The region distal to the BL (arrowhead) remains at this stage. (g and h): The wings at 2 days after pupation (P-2). The wing degeneration has completed at this stage. (h) Magnified view of the boxed area in (g). Scales have formed and long scales (arrowhead) are visible. In this figure and Figs. 2-4, distal is to the right and anterior is to the top. Bars: 1 mm. After emergence, female wings do not inflate. We observed that the size of adult wings is same as the degenerated wings at the late pupal stages, and therefore reduction of the female wings occurs in the early pupal stages.

size at an early pupal stage.

To study the developmental profile of female wing degeneration, we dissected developing wings during early pupal stages and observed the distribution of nuclei stained with methyl green (Fig. 1B). Immediately after pupation (P-0) and 24 hours after pupation (P-1), no morphological changes were observed and the cells in regions both distal and proximal to the BL existed (Figs. 1B, P-0 and P-1.5). Thirty-six hours after pupation (P-1.5), the female wing area had shrunk dramatically, although the BL structure still remained at the periphery (Figs. 1B, P-2). The male wing showed no such change at the same stage (data not shown). 48 hours after pupation (P-2), female wings had decreased to about one-fifth their original size and long scales had developed concurrently with the peripheral degeneration distal to the BL (Fig. 1B, P-2). Only peripheral degeneration was observed in male wings at the same stage (data not shown). These results indicate that peripheral degeneration outside the BL occurs on the same developmental schedule in both male and female wings, but the shrinkage of the entire wing region occurs simultaneously only in females.

Induction of female-specific wing degeneration by ecdysteroid

A previous study has demonstrated that peripheral degeneration in the wings of the silkworm is caused by an increasing titer of ecdysteroid during early pupal development (Fujiwara and Ogai, 2001). The above results suggest the possibility that the female-specific wing shrinkage that occurs concurrently with peripheral degeneration in *O. recens* is also induced by ecdysteroid. To test this hypothesis, we cultured pupal wings from both sexes, taken just after pupal ecdysis, and studied the effects of ecdysteroid on wing morphogenesis.

Female wings cultured in Grace's medium with 1 µg/ml 20E for two days (Fig. 2a) degenerated to approximately one-fifth the size (The cultured wings were left in their cuticular pouch. Compare the size of tissue with the cuticular pouch). Male wings cultured with 1 µg/ml 20E for two days (Fig. 2b) or for five days (data not shown) showed only peripheral degeneration, as observed in other Lepidoptera (Fujiwara and Ogai, 2001). Both female and male wings cultured without 20E showed no morphological change;

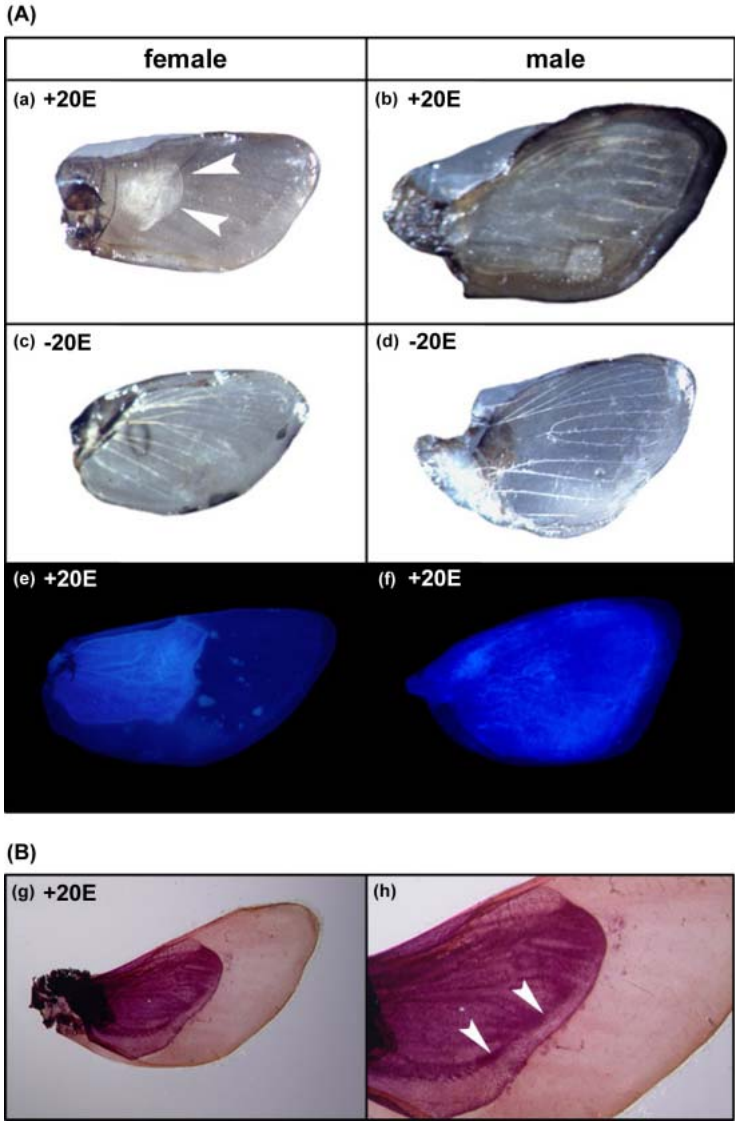

Figure 2. Morphological changes in cultured pupal wings induced by ecdysteroid (20-hydroxyecdysone, 20E). All wings were laid within their cuticular pouch. (A) (a) Female wing cultured with 1 µg/ml 20E degenerated to one-fifth the size (arrowheads). (b) Male wing cultured with 1 µg/ml 20E showed only peripheral degeneration. (c) Female wing cultured without hormone. (d) Male wing cultured without hormone. (e) Female wings cultured with 1 µg/ml 20E for two days and stained with DAPI. (f) Male wing cultured with 1 µg/ml 20E for two days and stained with DAPI. (B) Female wings cultured with 0.1 µg/ml 20E for 2 days and stained with Nuclear Fast Red (g). (h) Magnified view of (g). Note that the peripheral structure distal to the BL (arrowheads) remains in this condition.

the regions which cells exist were the same (Figs. 2c and 2d).

We next examined the dose-effect of ecdysteroid on female-specific wing degeneration (Table 1). Concentrations of 20E from 0.1 to 10 µg/ml induced shrinkage of the female pupal wing. A lower concentration of 20E (0.1 µg/ml) required a longer time to complete the degeneration processes. A higher dose of 20E (5 to 10 µg/ml, which is expected be higher than the hemolymph titer in pupa [Bollenbacher *et al*., 1981]), accelerated the degeneration. As shown above, female-specific degeneration also occurred over two days (from P0 to P2) in the developing pupae, consistent with results in the culture experiments. At 0.05 µg/ml of 20E, two of five pupae

Table 1. The dose effect of ecdysteroid on female-specific wing degeneration

20E (µg/ml)	0	0.05	0.1	0.5	1	5	10
Time (day)	-	3.5*	3	2	2	1.5	1.5
E (µg/ml)	0	0.05	0.1	0.5	1	5	10
Time (day)	-	-	4	3	3	2.5	2

Female pupal wings were dissected from pupae just after pupation and cultured with various doses of 20-hydoroxyecdysone (20E) and ecdysone (E). Morphological changes were observed with a binocular microscope. For each ecdysteroid concentration, 5-10 wings were tested. The time (in days) required for complete wing degeneration in average is shown in the table.
*: Two of five pupa tested showed no morphological changes.

tested showed no morphological changes. This indicates that the minimum concentration of 20E required for degeneration is approximately 0.05 µg/ml.

To observe the degenerated area more closely, we stained nuclei of degenerated wings. Figures 2e and 2f show DAPI staining in female and male wings cultured with 1 µg/ml 20E. Nuclei were located in the shrunken region of female wings, but were found throughout the male wing except the peripheral region. Female wings cultured with 0.1 µg/ml 20E (Fig. 2B, g and h) showed that the shrunken wings still retained the BL and the region distal to the BL. These results are consistent with morphological changes observed in the developing pupa, and clearly demonstrated that female-specific wing degeneration is induced by ecdysteroid.

Detection of Apoptotic Cells by TUNEL Analysis

Chromatin condensation and DNA fragmentation are early, diagnostic events during apoptosis. DNA fragmentation can be detected by labeling the ends of DNA fragments by the TUNEL method. We used this method to verify the area in which programmed cell death occurs in female wings (Fig. 3). Strong apoptotic signals were detected broadly in the wing margins, which corresponds exactly to the distal regions of the BL in the developing pupal wings of both males and females in P-0 (Figs. 3a and 3b). In developing pupae, however, apoptotic signals were not detected in regions other than the wing margin. To detect the initial apoptotic response induced by ecdysteroid more effectively, we next used TUNEL analysis with fluorescence microscopy to examine wings cultured with 20E for 6 hours. In male wings, TUNEL signals were detected only in the distal region of the BL (Fig. 3d). In contrast, small signals were dispersed throughout the region proximal to the BL in female wings (Fig. 3c). The sizes of these signals varied, and the largest seemed to correspond in size to hemocytes. Most of the large signals in female wings appeared to be fragmented nuclei trapped by the phagocytotic actions of hemocytes, and were not observed in epidermal cells. No signals were observed in male and female wings cultured without 20E for 6 hours (Figs. 3e and 3f).

Histological observation of female-specific degeneration in cultured wings

To observe the events that occur in the degenerated area, we next prepared semi-thin sections from cultured wings and studied the region-specific and sex-specific cellular responses under a light microscope. Just after pupation (P-0) and before culture, a small number of hemocytes were floating within the bi-layer of the wing epithelial cells or were attached to the epithelial cells both in females

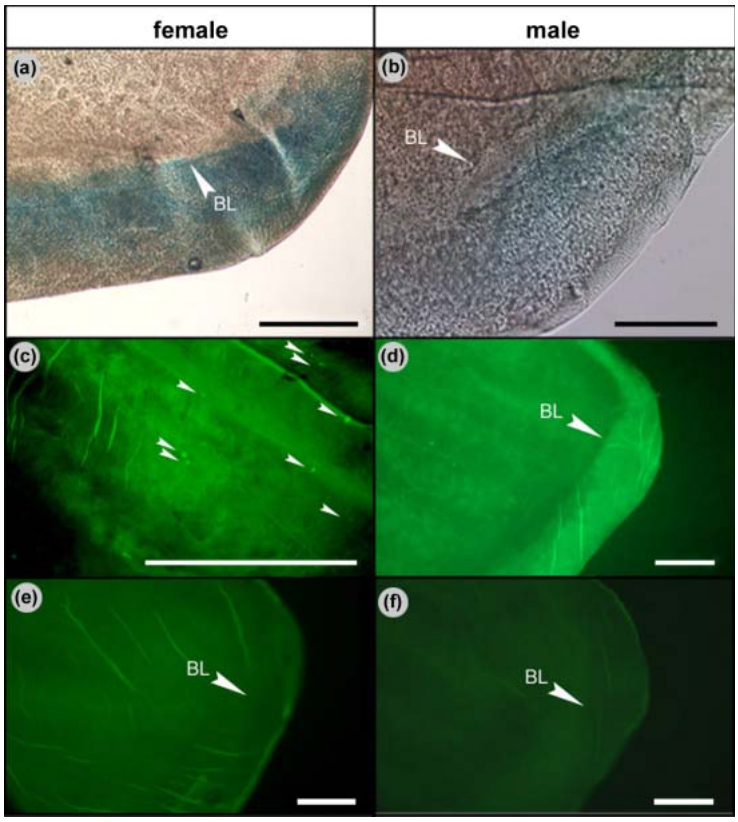

Figure 3. TUNEL detection of apoptosis in *O. recens* wings. (a) Female wing at 3 h after pupation. Blue signals (indicating apoptotic cells) were seen in the area distal to the BL (white arrowhead). (b) Male wing at 6 h after pupation. Blue signals were seen in the area distal to the BL. (c) Female wing cultured for 6 h with 20E. Apoptotic features (light green indicated by small white arrowheads) were visible in areas both distal and proximal to the BL. (d) Male wing cultured for 6 h with 20E. Light green signals were visible only in the area distal to the BL. (e) Female wing cultured for 6 h without 20E. No signals observed. (f) Male wing cultured for 6 h without 20E. No signals. Bars: 1 mm.

(Fig. 4A, P-0) and males (data not shown). Twenty-four hours after the addition of 20E to the female wing culture, the wing epithelia detached from the cuticle (apolysis), although no morphological change in wing shape was observed under a binocular microscope (data not shown). At this stage, the number of hemocytes increased, and some of these invaded the epithelial sheet in the proximal region of the BL (Fig. 4A, 24 h). Many plasmatocytes and granulocytes were identified by their morphology (red and yellow arrowheads). After 38.5 h of culture with 20E, the female wing degenerated to about 80% of its original size, although the region distal to the BL remained intact. At this stage, many hemocytes invaded the epithelial sheets, which seemed to trigger phagocytosis of the wing epithelia, in the regions both distal and proximal to the BL (Fig. 4A, 38.5 h). After 48 h of culture with 20E, all cells in distal region of the BL, and most epithelial cells in the proximal region of the BL had disappeared, and large hemocytes and a fibrous structure remained (Fig. 4A, 48 h). At this stage, the degenerative events were complete.

Male wings cultured with 20E for 38.5 h displayed different cellular features (Fig. 4B). The distal region of the BL had already disappeared and several hemocytes were left at the tip. Most of the hemocytes in the proximal region of the BL, which was detached from the wing epithelia, remained the same as they were before the addition of 20E. These observations indicate that the activity of the phagocytotic hemocytes in the region proximal to the BL clearly differs in male and female wings.

Discussion

In this study, we showed that female-specific wing degeneration in *O. recens* was induced by ecdysteroids (20E and E) in pupal wing culture. When ecdysone (E) was added in the medium

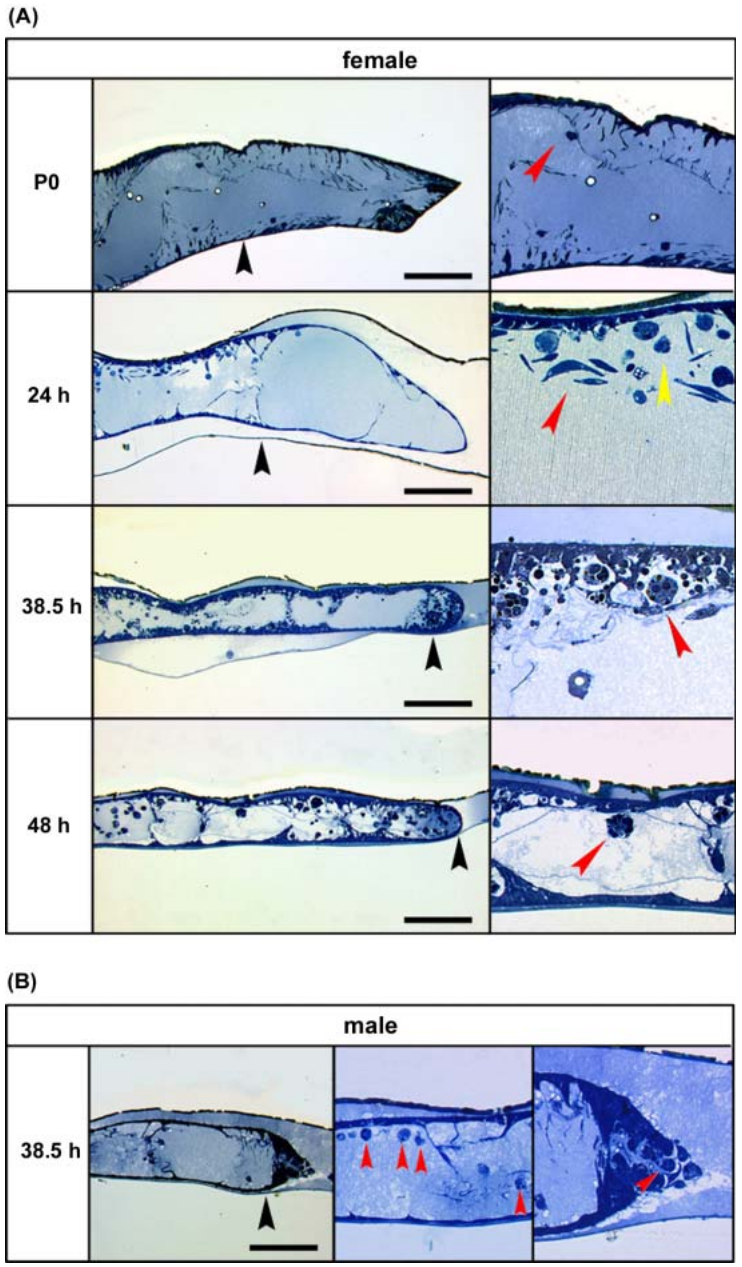

Figure 4. (A) Cross-sections of female wings cultured with 20E. Black arrowheads indicate the position of the BL. Female P-0: P-0 female wing; floating hemocytes are visible (right photo, red arrowhead). Female 24 h: wing cultured for 24 h with 20E; plasmatocytes (right photo, red arrowhead) and granulocytes (right photo, yellow arrowhead) are visible in the area proximal to the BL. Female 38.5 h: wing cultured for 38.5 h with 20E. Phagocytosis (right photo, red arrowhead) are seen in areas both distal and proximal to the BL. Female 48 h: hemocytes are visible floating in the wing (right photo, red arrowhead). (B) Male 38.5 h: male wing cultured for 38.5 h with 20E; phagocytosis was observed only in the area distal to the BL (right photo, red arrowhead). Bars: 100μm.

instead of 20E, female-specific wing degeneration was induced less effectively (Table 1). This observation is consistent with the fact that E is the precursor of 20E and is less efficacious in various biological activities in most insects. Because the culture medium included only inorganic substances and no morphological change was observed without 20E, we propose that ecdysteroid is the key factor driving these female-specific events. The differential development of wings in the males and females seems to be contingent on ecdyteroids that cause sex-specific changes in downstream regulatory events affecting apotosis of wings.

Peripheral degeneration in the distal region of the BL of pupal wings is commonly observed in most Lepidoptera. The adult wing border of Lepidoptera is defined by the position of the BL (Kodama *et al*., 1995). The intensive cell death induced by ecdysteroid occurs in the region distal to the BL (Fujiwara and Ogai, 2001). Therefore, we originally hypothesized that the BL of female wings of *O. recens* lies near the base of the wing, which would cause the excessive reduction in female wing size. However, the BL in the female wing runs near and parallel to the outline of the wing, similar to that of the male (Figs. 1Ae and 1Af). Cell death is present throughout entire wings in the female at P-1.5; the distal region of the BL is completely degenerated at P-2 (Fig. 1B). This indicates that the mechanisms for peripheral degeneration are conserved among the Lepidoptera, and that the female-specific cell death has been introduced into *O. recens* as an additional developmental event during evolution.

TUNEL analysis and histological observation showed that both apoptotic events and phagocytotic activation were also ecdysone-inducible and gender-specific (Figs. 3 and 4). In cultured wings of *O. recens* females, apoptotic traits were detected throughout the entire wing, and were induced by 20E (Fig. 3c), but did not occur in the absence of 20E (Fig. 3e). The number of hemocytes that engulfed the apoptotic cells was also enhanced throughout the entire wing area of the female by treatment with 20E (Fig. 4A). It is known that hemocytes play dual roles in development and in immune defense (Kiger, *et al*., 2001, Pace, *et al*., 2002). They are formed in lymph glands and differentiate into 5 types of hemocytes in moths (Willott, *et al*., 1994). In *Manduca sexta*, some types of hemocytes are involved in morphogenesis of basal lamina, which seems correlated with the rise of ecdysteroid level (Nardi, *et al*., 2000). In addition, Lanot *et al*. (2001) observed that the hemocytes differentiate by injection of 20E into mid-third instar larvae of *Drosophila melanogaster*. These facts support the idea that 20E also acts on differentiation and proliferation of hemocytes in *O. recens* wings. In the wing *in vitro* culture system used in this study, hemocytes can no longer move into the wings from the pupal body, and therefore hemocytes pre-existing within the wings may have been activated to differentiate and to become phagocytotic by the addition of 20E. In contrast to the wings of *O. recens* females, 20E-induced apoptotic characteristics and phagocytotic activation were restricted to the region distal to the BL in male wings. Phagocytotic hemocytes can usually discriminate between apoptotic wing epithelial cells and non-apoptotic cells (Green and Beere, 2000). Therefore, the restricted localization of hemocytes may reflect the region-specific apoptosis induced by ecdysteroid.

The ecdysteroid signal should be received in female wings, because female-specific wing degeneration in *O. recens* is also an ecdysteroid-dependent event. The different cellular responses in pupal wings described above may be explained by the region-specific expression of specific ecdysone receptor (*EcR*) isoforms, which govern distinct ecdysteroid-induced responses in differentiating cells of various insects (Talbot *et al*., 1993, Riddiford *et al*., 2000). It was recently reported that the localization of EcR proteins in butterfly wings correlated to color pattern formation and the development (Koch PB *et al*., 2003). However, preliminary results showed no differences in the expression patterns of EcR isoforms in the male and female wings of *O. recens,* at least immediately after pupal ecdysis (data not shown), although we did not examine the patterns in other stages. The EcR molecule functions as a heterodimer with ultraspiracle protein (USP), and its function is sometimes modulated by another partner, such as hormone receptor 3 (DHR3) (Lam, *et al*., 1999), Seven-up (Svp) (Kerber *et al*., 1998) or Broad Complex (BRC) (Zhou *et al*., 1998). Therefore, another possible explanation for the effects of ecdysteroid on wing development of female *O. recens* involves an alteration of the EcR partner in the EcR-USP heterodimer. Differential development could be due to differences in any aspects of downstream signaling even if the receptor characteristic did not differ between male and female. Further studies will clarify which molecules are responsible for ecdysteroid-dependent sexual dimorphism formation. The results described above provide a significant clue not only to the hormonal and developmental regulation of wing polymorphism of *O. recens* through ecdysteroid-driven pathway, but also to the sexual differentiation processes during metamorphosis in insects at the molecular level.

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