The Journal of Experimental Biology 207, 1151-1161
Published by The Company of Biologists 2004
doi:10.1242/jeb.00855

Overexpression of *broad*: a new insight into its role in the *Drosophila* prothoracic gland cells

Xiaofeng Zhou, Baohua Zhou*, James W. Truman and Lynn M. Riddiford†

Department of Biology, University of Washington, Box 351800, Seattle, WA 98195-1800, USA

*Present address: Virginia Mason Research Center, 1301 Ninth Avenue, Seattle, WA 98101-2795, USA

†Author for correspondence (e-mail: lmr@u.washington.edu)

Accepted 28 December 2003

Summary

Insect molting is triggered by ecdysteroids, which are produced in the prothoracic glands (PG). The *broad* (*br*) gene is one of the 'early genes' directly regulated by ecdysteroids. Ectopic expression of the BR-Z3 isoform in early second instar *Drosophila* larvae (L2) before the rise of the ecdysteroid titer prevented molting to the third instar, but the larvae subsequently formed L2 prepupae after prolonged feeding. When these larvae were fed on diet containing 20-hydroxyecdysone (20E), they formed pharate third instar larvae. The critical weight for normal L3 pupariation of w^{1118} larvae was found to be 0.8 mg and that for L2 pupariation was 0.45 mg. We also defined a threshold weight for metamorphosis of 0.3 mg, above which L2 larvae will metamorphose when provided with 20E. BR-Z3 apparently works through the PG cells of the ring gland but not the putative neurosecretory cells that drive ecdysone secretion, because ectopic expression of BR-Z3 specifically in the ring gland caused 53% of the larvae to become permanent first instar larvae. Driving other BR isoforms in the ring gland prevented larval molting or pupariation to varying degrees. These molting defects were rescued by feeding 20E. Overexpression of each of the BR isoforms caused degeneration of the PG cells but on different time courses, indicating that BR is a signal for the degeneration of the PG cells that normally occurs during the pupal–adult transition.

Key words: *broad*, critical weight, ecdysteroid, metamorphosis, molt, prothoracic gland, *Drosophila*.

Introduction

Molting and metamorphosis of larval insects is intimately associated with growth. Typically, the larva must undergo a minimal amount of growth before it can molt to the next larval stage. Also, a critical weight must be achieved before the larva can begin metamorphosis (Nijhout, 1981, 1994). These growth-related decisions interact with the neuroendocrine systems that control molting and metamorphosis. The decision to molt is mediated through the prothoracicotropic hormone (PTTH) that is released from the brain to evoke ecdysteroid biosynthesis and release from the prothoracic gland (PG). The critical weight for metamorphosis, by contrast, is associated with the decline of juvenile hormone (JH) from the corpora allata (CA), which is necessary for metamorphosis to occur. In *Drosophila*, the PG cells that secrete ecdysone (Kiriishi et al., 1990) are part of the ring gland, which also contains the CA and the corpus cardiacum (CC; a neurohemal organ).

The products of the *broad* gene (*br*; previously called the *Broad-Complex* or *BR-C*) are ecdysteroid-inducible transcription factors that are involved in pupal commitment at the onset of metamorphosis (Zhou et al., 1998; Zhou and Riddiford, 2001, 2002). In *Drosophila*, *br* is among those few 'early genes' that respond directly to the ecdysteroids. These primary response genes, including *br*, *E74* and *E75*, regulate several secondary response genes, which in turn direct appropriate biological responses to each ecdysteroid pulse during development (Russell and Ashburner, 1996; Thummel, 1996, 2002; Baehrecke, 2000). The proteins encoded by *br* are members of the Broad-Tramtrack-Bric-a-brac (BTB) family of transcription factors that share a common N-terminal domain thought to be important in protein–protein interactions (Zollman et al., 1994). The alternately spliced C-terminus contains one of four pairs of C_2H_2 zinc fingers that putatively bind DNA (DiBello et al., 1991; von Kalm et al., 1994; Bayer et al., 1996; but see Mugat et al., 2000). The *br* gene is defined by three genetic functions: *broad* (*br*), *reduced bristles on palpus* (*rbp*) and *2Bc* (Belyaeva et al., 1980; Kiss et al., 1988). The *rbp* genetic function is provided by the BR-Z1 isoform and partially by Z4, the *br* allele by Z2, and *2Bc* by Z3 (Bayer et al., 1997). Mutants devoid of all isoforms develop normally to the final larval instar but fail to form a normal puparium, indicating that *br* is required for metamorphosis (Kiss et al., 1976, 1988).

In *Drosophila*, all the tissues express Broad (BR) proteins during metamorphosis, but there is both temporal and tissue specificity as to the predominant isoform present (Huet et al., 1993; Emery et al., 1994; Bayer et al., 1996; Mugat et al., 2000;

Brennan et al., 2001; Ghbeish et al., 2001). We have shown previously that the *br* gene products, especially the Z1 isoform, can induce the expression of pupal-specific cuticle genes and suppress both larval and adult cuticle genes in epidermal cells during a molt, thus causing a pupal molt (Zhou and Riddiford, 2002). Recently, *br* was also found to be necessary for programmed cell death in the salivary glands; the maximal expression of the death genes *head involution defective* (*hid*) and *reaper* (*rpr*) require *br* function (Jiang et al., 2000). Additionally, BR proteins directly upregulate the caspase DRONC, which is an initiator caspase essential for programmed cell death (Cakouros et al., 2002).

During our previous studies, we demonstrated that misexpression of the different BR isoforms by heat-shock induction of their transgenes during the late second instar disrupted normal third instar cuticle production during the molt (Zhou and Riddiford, 2002). Unexpectedly, in these studies we found that premature expression of the Z3 isoform early in the second instar suppressed the molt to the third instar and resulted in larvae forming miniature puparia after an extended feeding period. In the present study, we find that the presence of BR in the PG cells is responsible for this repression of larval molting. We also demonstrate, for the first time, that BR proteins are involved in the degeneration of the PG cells during metamorphosis.

Materials and methods

Drosophila culture and stocks

All flies were reared at 25°C on standard cornmeal–molasses-based medium (Sullivan et al., 2000). For staging, embryos were collected at 30-min intervals, and the larvae were resynchronized at ecdysis into the second or the third instar.

The following transgenic fly lines carrying one of the BR isoform cDNAs under the control of the heat-shock promoter were used: w^{1118}*; 527-5; 708-1* (four copies of *hs-BR-Z1*), w^{1118}*; cD5-4C; cD5-1* (four copies of *hs-BR-Z2*), w^{1118}*; 797-3; 797-E8* (four copies of *hs-BR-Z3*) and w^{1118}*; Z4-13; Z4-1* (four copies of *hs-BR-Z4*) (Crossgrove et al., 1996; Bayer et al., 1997). w^{1118} was used as control. To overexpress BR proteins, transgenic lines were heat shocked in vials for 30 min at 37°C in a water bath at the beginning of the designated time.

Enhancer trap lines expressing GAL4 in the PG cells of the ring gland were P0206 and P0163 from Dr W. Janning (http://FlyView.uni-muenster.de), and those expressing Gal4 in the putative PTTH-producing cells were Feb 211 and Feb 204 (Siegmund and Korge, 2001). When these lines were used, the embryos were collected at 2-h intervals.

Transgene constructs and germline transformation

To construct *UAS-BR* isoform transgenic lines, the full-length cDNAs encoding each of the four Z isoforms were cloned individually into the P[UAST] vectors between the *Eco*RI and *Xho*I sites (Brand and Perrimon, 1993). These constructs were used in germline transformation as described (Rubin and Spradling, 1982).

Starvation and critical weight

For analysis of the critical feeding period necessary for a second-to-third instar molt, newly ecdysed second instar larvae were allowed to feed on the standard cornmeal–molasses diet for a designated time at 25°C, then were moved to a protein-free diet containing only 10% D-glucose and 3% agar. The animals were scored for molting at 24–28 h after the ecdysis into the second instar.

For analysis of the critical weight for pupariation, larvae were weighed individually to the nearest 0.002 mg on a microbalance (Mettler M5). The individual was then starved in a vial with wet tissue, and pupariation was scored.

20E feeding experiments

To provide exogenous ecdysteroid, yeast paste or fly diet containing 1 mg ml^{-1} 20-hydroxyecdysone (Sigma, St Louis, MO, USA) in 5% ethanol was used.

Immunostaining and microscopy

Drosophila larvae were cut open along the dorsal middle line in phosphate-buffered saline (PBS) and then fixed in 3.7% formaldehyde and processed as previously described (Zhou and Riddiford, 2001). Primary antibodies used were anti-BR core region monoclonal antibody (mAb) at 1:250 dilution (Emery et al., 1994), and polyclonal antibodies for BR-Z1, -Z2, -Z3 and -Z4 isoforms at 1:3000 dilution (Mugat et al., 2000). Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit antibody and Texas Red-conjugated donkey anti-rabbit at 1:500 dilution (Jackson ImmunoResearch, West Grove, PA, USA). Fluorescent visualization was with a BioRad MRC-600 confocal laser scanning microscope and images were processed with NIH Image and Adobe Photoshop.

Photos of prepupae and pharate adult flies were taken with a Cool Snap camera on a Wild dissecting microscope and processed with RS Image software. Images of whole mounts of mouthhooks were captured with a Sony video camera on a Nikon Optiphot microscope and processed with Adobe Photoshop.

Results

Formation of L2 prepupae after misexpression of BR-Z3 during the early second larval instar

Using heat-inducible transgenic fly lines of each BR-Z isoform (Crossgrove et al., 1996; Bayer et al., 1997), we previously demonstrated that misexpression of the Z1 isoform during the mid-second instar caused premature expression of the pupal cuticle gene *Edg78E* (Zhou and Riddiford, 2002). Expression of other isoforms, by contrast, had little effect on *Edg78E*. All these animals formed a new cuticle as evidenced by double mouthhooks and double spiracles (Fig. 1A) but they failed to ecdyse as third instar larvae. Surprisingly, when four copies of BR-Z3 were induced by heat shock within 5 h after ecdysis to the second instar, 83% of the larvae (N=210) failed to initiate the third instar molt but continued to eat and grow,

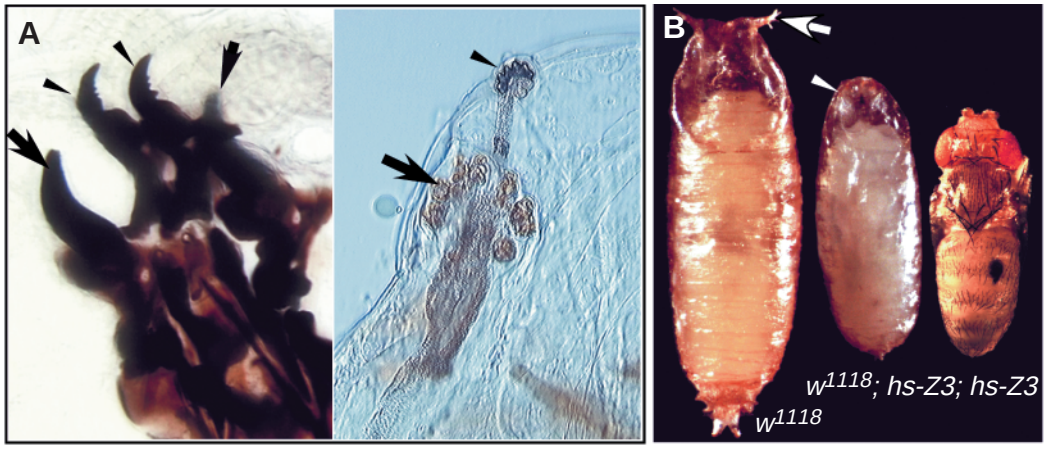


Fig. 1. Effects of overexpression of BR-Z3 in w^{1118}; *hs-Z3; hs-Z3* larvae. (A) Induction of BR-Z3 at 11 h after ecdysis to the second instar. Left: blocked pharate third instar larva showing both the second instar (arrow heads) and the third instar (arrows) mouthhooks. Right: double spiracles in a blocked pharate third instar larva (arrow head points to the second instar spiracle, and arrow points to that of the third instar). (B) Induction of BR-Z3 at 5 h after ecdysis to the second instar. Left: normal third instar puparium. Middle: second instar puparium after experimental treatment. Note the uneverted anterior spiracles (arrow head) in the L2 puparium as compared with the everted multi club-shaped third instar spiracles (arrow). The L2 puparium is about two-thirds the size of the w^{1118} normal puparium. Most of the L2 pupae developed up to stage P8 and, in very rare cases, they formed pharate adults (right).

far exceeding the size of a normal second instar larva. Eventually, these second instar larvae pupariated at ~93 h after the heat-shock treatment, forming what will be referred to hereafter as L2 prepupae (Fig. 1B). The L2 prepupae are characterized by their uneverted, single club-shaped anterior spiracles and small mouthhooks with a few teeth, characteristic of the second instar larva (Fig. 1; Bodenstein, 1965). Approximately 97% of the L2 prepupae (*N*=125) successfully everted their imaginal discs and head, and most of them developed up to stage P8, showing pale yellow pigmentation in the eyes (Bainbridge and Bownes, 1981). In very rare cases, they became pharate adults (Fig. 1B).

In contrast to the effects of BR-Z3 expression, few or no L2 prepupae were formed after misexpression of the other BR isoforms during the early second instar (Table 1). Overexpression of BR-Z1 at this time caused the death of the animal at ~42–68 h after the heat shock with no detectable third instar mouthhooks (*N*=150). Expression of either BR-Z2 or -Z4 caused a high rate of death at ~26 h of the second instar, the time of ecdysis from the second to the third instar (*N*=135 and 107, respectively). Double mouthhooks were seen in these dead larvae.

To determine the BR expression pattern after the heat shock, we used a mAb against the BR protein core region (Emery et al., 1994). The BR protein appeared globally in all the tissues up to and including 12 h after the heat shock and then gradually disappeared (Fig. 2A). At 18 h, only trace amounts remained in the fat body (Fig. 2B), and by 24 h no BR protein was seen in any tissue except the central nervous system (CNS; Fig. 2C). The BR staining in the CNS, however, was due to the endogenous Z3 protein, since in wild-type and w^{1118} larvae, the CNS normally expresses the Z3 isoform in late embryogenesis through larval life (Fig. 2F; Zhou, 2000; B.Z., D. Williams, L.M.R. and J.W.T., unpublished). By 36 h, BR began to reappear in the tissues of these persisting second instar larvae (Fig. 2D,E). This later round of expression of *br* matches the tissue-specific expression seen during the normal onset of metamorphosis in the mid-to-late third instar (Crossgrove et al., 1996; Mugat et al., 2000).

Relationship between the critical feeding period and the effects of BR-Z3 misexpression

Based on the above observations, we hypothesized that misexpression of BR-Z3 before the synthesis and release of ecdysteroids in the second instar prevented the later rise of the ecdysteroid titer, so that the larvae did not molt but continued to eat and grow. Then, once these L2 larvae reached a critical weight, the metamorphic program was initiated, leading to the formation of L2 prepupae. To test this hypothesis, we determined the critical weight necessary for the second-to-third instar larval molt and the period when misexpression of BR-Z3 could induce the formation of L2 prepupae.

After molting to a given larval instar, many insect larvae must achieve a certain critical weight before they can initiate the molt to the next instar (Truman, 1972; Nijhout, 1981; Gilbert et al., 2002). To determine whether a similar critical weight exists for the molt from the second to third instar in

Table 1. *Percentage forming L2 prepupae after misexpression of different BR-Z isoforms at 5 h after ecdysis to the second instar*

	BR-Z isoform			
	Z1	Z2	Z3	Z4
% of L2 prepupae (*N*)	0 (150)	7 (135)	83 (210)	12 (107)

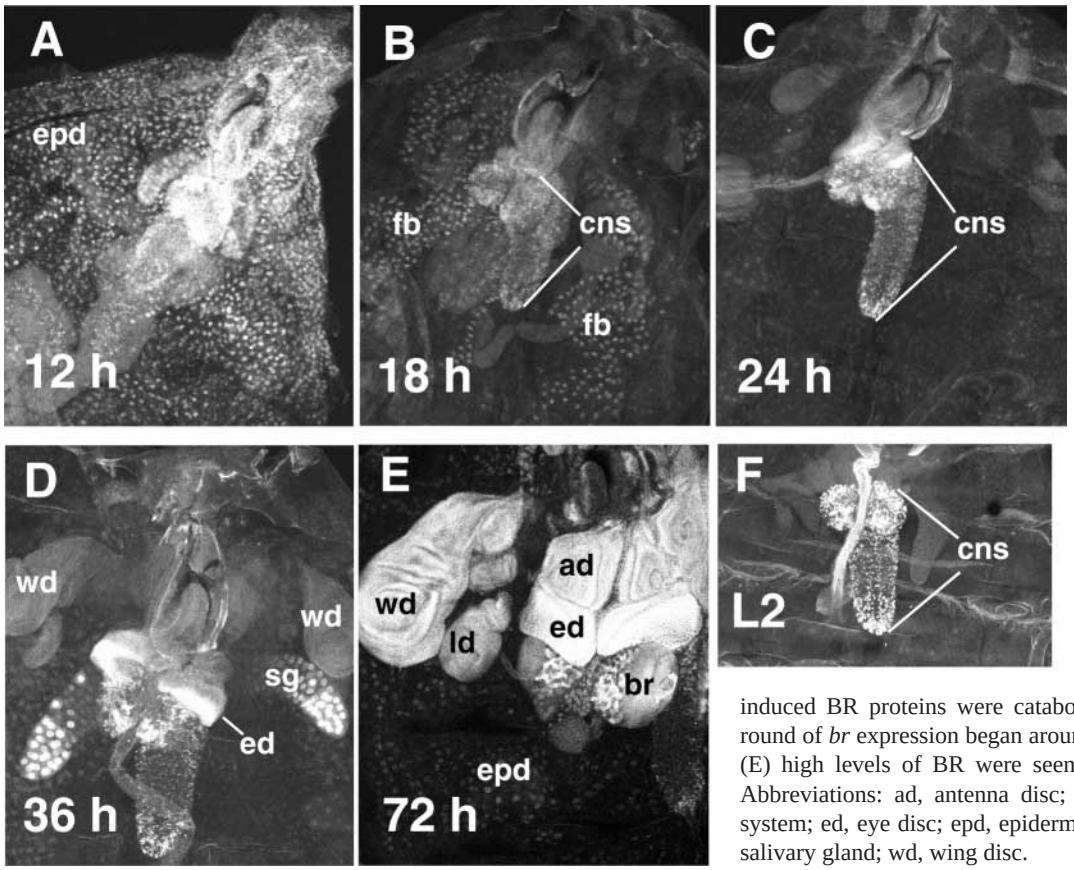
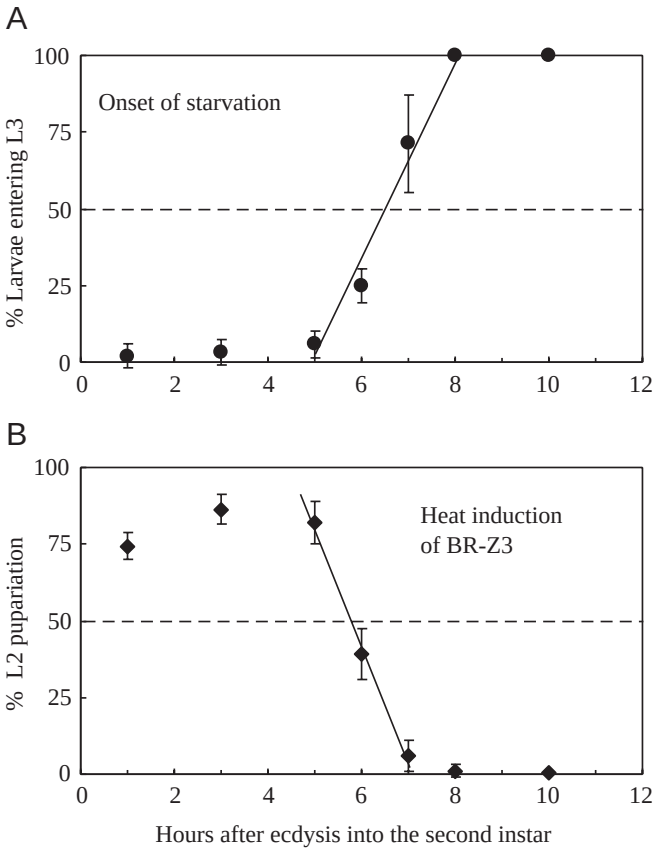


Fig. 2. Broad (BR) protein expression pattern after heat induction of BR-Z3 at 5 h of the second instar. (A) BR proteins were detected in all tissues by an anti-BR core region antibody at 12 h after the heat shock. (B) At 18 h only trace amounts of BR protein remain in the fat body. BR is also found in certain cells in the CNS, as in normal second instar larvae (F). (C) By 24 h after the heat shock, BR proteins were only detected in the CNS, as in the normal second instar larva (F), indicating that all heat-induced BR proteins were catabolized by that time. (D) A new round of *br* expression began around 36 h after the heat shock, and (E) high levels of BR were seen in the imaginal discs at 72 h. Abbreviations: ad, antenna disc; br, brain; cns, central nervous system; ed, eye disc; epd, epidermis; fb, fat body; ld, leg disc; sg, salivary gland; wd, wing disc.



Drosophila, we allowed newly ecdysed second instar larvae to feed on standard cornmeal–molasses diet for various times, then transferred them to a diet containing only glucose. The glucose diet supported the energetic requirements of the larvae but not further growth. The animals were then scored for molting at 24–28 h after ecdysis into the second instar. Only ~5% of second instar larvae that were 5 h old or younger at the time of the switch to the glucose diet were able to molt to the third instar (Fig. 3A). By contrast, 71% of 7-h-old larvae and all animals older than 8 h at the time of the switch successfully ecdysed to the third instar at the normal time. The critical feeding period for the second-to-third instar molt as defined by

Fig. 3. Overexpression of BR-Z3 during the critical feeding period for the second-to-third instar molt causes a prolonged second instar and the formation of L2 prepupae. (A) Second instar larvae were allowed to feed on standard diet for a designated period and were then transferred to glucose diet (protein starvation). Ecdysis to the third instar was scored. Each point (circle) represents a mean of four repetitions (N=16, 8, 8, 8) ± standard deviation (S.D.). Note the ET_{50} for the critical feeding period is ~6.5 h. (B) BR-Z3 was overexpressed by a heat shock at a designated time in the second instar, and the animals were maintained on the standard diet. The L2 prepupae were scored. Each point (diamond) represents a mean of four repetitions (N=25, 10, 10, 10) ± S.D. Note that the ET_{50} of the induction of L2 prepupae is ~5.8 h after ecdysis into the second instar.

the 50% effective time (ET_{50}) was ~6.5 h (Fig. 3A). By that time, the larvae had attained a weight of 0.15 mg on average (N=46). This weight then is the critical weight that the larva must achieve before it can initiate the molt to the third larval instar.

As seen in Fig. 3B, heat-shock induction of BR-Z3 at any time within the first 5 h of the second instar caused most larvae (>74%, N=55) to continue to eat and grow without entering the third instar, and these larvae eventually formed L2 prepupae. Ectopic expression of BR-Z3 in second instar larvae that were 7 h or older, by contrast, did not prevent forming the pharate third instar, although most did not ecdyse. The ET_{50} for induction of the formation of L2 prepupae was 5.8 h after entering the second instar (Fig. 3B). This time point is slightly earlier than the critical feeding period for the second-to-third instar molt, which is probably explained by the lag between heat shock and the appearance of the BR-Z3 protein. Our data strongly suggest that ectopic expression of BR-Z3 before the initiation of the endocrine events for the third instar larval molt prevents the normal molting rise of the ecdysteroid titer.

The effect of 20E on the BR-Z3 induced prolonged second instar larvae

If the prolonged second instar caused by misexpression of Z3 was indeed due to the suppression of the larval ecdysteroid rise, one should be able to rescue the larval molt by giving exogenous 20E to these animals. We therefore fed 20E to the second instar larvae expressing BR-Z3 after heat-shock treatment. In all the subsequent experiments, BR-Z3 was induced by a heat shock at 5 h after ecdysis to the second instar. After the heat shock, the larvae were transferred to yeast paste containing 1 mg ml^{-1} 20E (referred to as '20E diet' hereafter). All of these larvae subsequently underwent a larval molt, indicating that 20E could initiate a larval molt despite the induced BR. This result shows that the second instar larval feeding period was probably prolonged due to the lack of ecdysteroids. Interestingly, if larvae were fed on regular diet for more than 20 h after the induction of BR-Z3 by the heat shock, then transferred to the 20E diet, most of them continued to eat and formed L2 prepupae after a similar time period as those continuously fed on regular diet (N=65). Thus, there is a time after which these larvae can no longer undergo a larval molt, even when challenged with 20E.

Feeding 20E to the second instar larvae in which BR-Z3 was induced early will cause them to molt to the third instar, but ecdysis was not successful and all died with double mouth hooks (N=129). This blockage at ecdysis is the same as seen when BR-Z3 is induced *after* the critical feeding period. The BR-Z3 protein induced by heat shock persisted for ~15 h in various tissues (Fig. 2). To determine if it was the presence of BR-Z3 that caused the lethality during the ecdysis to the third instar, we placed larvae on a glucose diet for 20–24 h after the heat shock. The BR-Z3 protein should completely disappear during this period (Fig. 2C) when the larvae are unable to grow (i.e. remain below the critical weight for pupariation). After transfer to the 20E diet, these larvae formed pharate third instar larvae but still died during ecdysis (N=91), indicating that the lethality was probably due to the downstream effects of globally expressed BR-Z3.

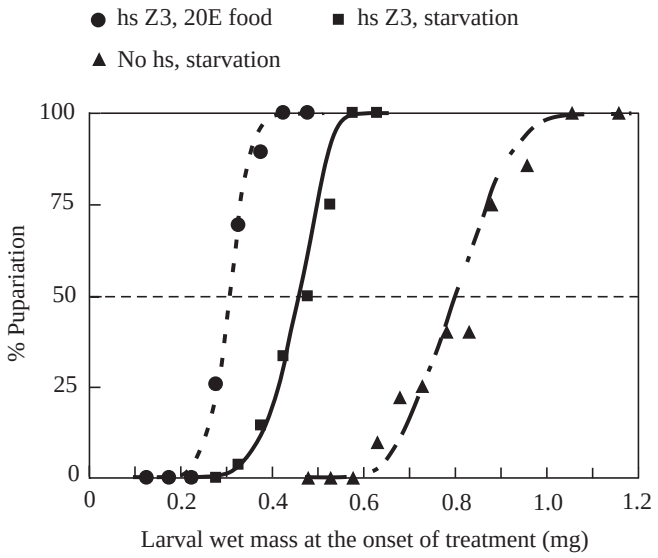


Fig. 4. Critical weights for pupariation for *w^{1118}; hs-Z3; hs-Z3* larvae. Larvae were raised on standard diet, then given (or not given) a heat shock at 5 h after ecdysis into the second instar. Individuals of various ages (16–35 h after the heat shock) were weighed, then were either starved or fed 20-hydroxyecdysone (20E) diet. Pupariation was scored for each group of treatment. The percentage of pupariation was calculated from groups of larvae whose weight was the designated point ± 0.025 mg. The number of animals used for measuring the critical weights for normal L3 pupariation (triangles), L2 prepupae (squares) and competence to form L2 prepupae (circles) was 80, 117 and 99, respectively.

Critical weight for L2 pupariation

To determine the critical weight necessary for L2 pupariation, L2 larvae that had overexpressed BR-Z3 at 5 h after ecdysis were collected at various times after the heat shock, weighed and held in moist vials without food. Siblings that had not experienced the heat-shock treatment served as controls. As seen in Fig. 4, these BR-Z3-expressing larvae showed a critical weight of 0.45 mg, with those larger than this size typically forming an L2 puparium. These BR-Z3-expressing larvae required ~32 h after the heat shock [i.e. 85 h after egg laying (AEL)] to attain this critical weight. The critical weight for L3 pupariation of the control siblings in this study was 0.80 mg, which was attained by ~7 h of growth in the third instar (i.e. 79 h AEL). A newly ecdysed third instar *w^{1118}* larva weighed 0.52 mg on average (N=40).

In view of the different critical weights that are used for L2 and L3 pupariation, we wished to determine whether there is a critical weight for forming L1 prepupae. When BR-Z3 was induced in early first instar larvae by heat shock at 2–5 h, none formed an L1 prepupa (N=60). Instead, these larvae ecdysed to the second instar but with an 18-h delay at 25°C. Heat induction of BR-Z3 at 4 h after hatching followed by two heat

shocks at ~16 h and ~28 h prevented 96% of the larvae (*N*=23) from molting to the second instar. These permanent first instar larvae continued feeding and grew for ~4 days but were unable either to molt to the second instar or to form puparia.

As previously noted, transfer of second instar larvae expressing BR-Z3 to the 20E diet caused either a larval molt or formation of L2 prepupae, depending on the time of transfer. As evident in Fig. 4, the switch in response to 20E corresponds to the time that the larvae reached 0.30 mg, which is 0.15 mg less than the critical weight (0.45 mg) determined by starvation. This lower weight (0.30 mg) defines the animal's competency to form a prepupa, since larvae above this weight responded to 20E by initiating metamorphosis. Therefore, we will refer to this weight as the 'threshold weight' for metamorphosis. The second critical weight (0.45 mg) determines when the endocrine events involved in the larval–pupal transition are initiated; after attaining this weight, a larva can pupariate without further feeding and on the same time schedule as larvae that continue to feed.

BR-Z3 protein affects functioning of the PG cells, not that of the putative PTTH cells

The data above suggest that the global expression of BR-Z3 may prevent the ecdysteroid rise in a time-restricted manner. However, these data do not show which tissue or cells mediate this action of BR-Z3. We therefore used the GAL4–UAS system to target the BR-Z3 expression in selected components of the neuroendocrine system. The PG cells of the ring gland are the predominant source of ecdysone, and the synthesis and release of ecdysone is assumed to be controlled by the brain neuropeptide PTTH (Gilbert et al., 2002). Tissue-specific GAL4 enhancer trap lines were used to express BR-Z3 in either the PG-LP neurons in the brain (the putative PTTH neurons) or the PG cells of the ring gland.

Two GAL4 enhancer trap lines were used to express BR-Z3 in the PG-LP neurons. Line Feb 211 has strong expression of GAL4 in the PG-LP neurons and weak expression in a brain lateral neurosecretory neuron (CC-LP 2), while Feb 204 weakly expresses GAL4 in the PG-LP neurons as well as in a few neurosecretory neurons of the medial subesophageal ganglion (CC-MS 1 and CC-MS 2) (Siegmund and Korge, 2001). When the GAL4 line Feb 211 was crossed with a UAS BR-Z3 line, their progeny underwent normal larval and pupal molts (*N*=57). However, these animals failed to eclose completely, with their abdomens remaining in the puparial cases. Similarly, when the GAL4 Feb 204 line was crossed with the UAS BR-Z3 line, most of the progeny formed puparia (78%, *N*=127). All the prepupae successfully pupated (*N*=55), ~18% of them arrested at stage P7, 12% arrested as pharate adults, 14% partially eclosed and 56% fully eclosed. Hence, ectopic expression of BR-Z3 in the putative PTTH-producing cells has no effect on larval molting or pupariation, although it does interfere with events late in the metamorphic molt.

To determine if BR-Z3 affects the ecdysteroid titer through effects on the PG cells, we crossed the UAS BR-Z3 line with two lines that express GAL4 in the PG cells and selected other tissues. Line P0206, which has a P element insertion upstream of *snail* (Eugenio Gutierrez and Alex Gould, personal communication), specifically expresses GAL4 in the ring gland (both the PG cells and the CA cells), salivary gland and oenocytes during later embryonic and larval stages (http://FlyView.uni-muenster.de; data not shown). Approximately 53% of the larvae (*N*=260) expressing BR-Z3 under control of this driver became permanent first instar larvae surviving for 4–6 days. Of those larvae that ecdysed to the second instar (*N*=47), 81% were permanently arrested in the second instar, only 19% ecdysed to the third instar and less than 1% formed L2 prepupae (Fig. 5C). When another enhancer trap line P0163, which also expresses GAL4 in the ring gland (http://FlyView.uni-muenster.de), was crossed with the UAS BR-Z3 line, 63% of the progeny (*N*=76) remained in the first or second instar (data not shown). These observations strongly suggest that misexpression of BR-Z3 affects the ecdysteroid titer through the ring gland, probably through the PG cells themselves.

Driving other BR-Z isoforms in the PG cells also blocks molting and can be rescued by feeding 20E

To investigate the effects of the other BR-Z isoforms on PG function, we used P0206 to drive each of the Z isoforms in the ring gland. Approximately 99% of larvae expressing BR-Z1 and 78% of larvae expressing BR-Z2 in the ring gland remained in the first instar (Fig. 5A,B). None of the second instar larvae expressing BR-Z2 molted to the third instar although 4% formed L2 prepupae (Fig. 5B). When the Z4 isoform was expressed in the ring gland, all eventually became third instar larvae, but only 8% pupariated (Fig. 5D).

To determine whether the defects in molting seen after expression of the BR-Z isoforms in the ring gland are due to the lack of ecdysteroid secretion, we fed 20E to these animals. Since our starvation experiments suggested that a critical feeding period of ~6.5 h is required for a larval molt (Fig. 3A), we fed 20E diet to these BR-expressing animals at ~8 h after hatching. These larvae molted to the second instar (Fig. 5). Continuing exposure to 20E diet, however, caused high lethality. When the freshly molted second instar larvae were placed on normal diet for 8 h, then transferred to 20E diet, most subsequently ecdysed to the third instar. When 20E was given to the 'rescued' third instar larvae after one day of feeding on normal diet, approximately 69%, 52%, 68% and 81% of the larvae misexpressing the BR-Z1, -Z2, -Z3 and -Z4 isoforms, respectively, pupariated (Fig. 5). When 20E-rescued second instar larvae expressing BR-Z1, -Z2 or -Z3 were placed on standard diet, they grew but remained in the second instar. When transferred to 20E diet after 2 days on standard diet, most of them formed L2 prepupae rather than third instar larvae (Fig. 5A–C), presumably because they had surpassed the critical weight for formation of L2 prepupae. Our data suggest that when any of the BR-Z isoforms are ectopically expressed in the PG cells, various degrees of ecdysteroid deficiency ensue, resulting in the cessation of a larval molt or pupariation.

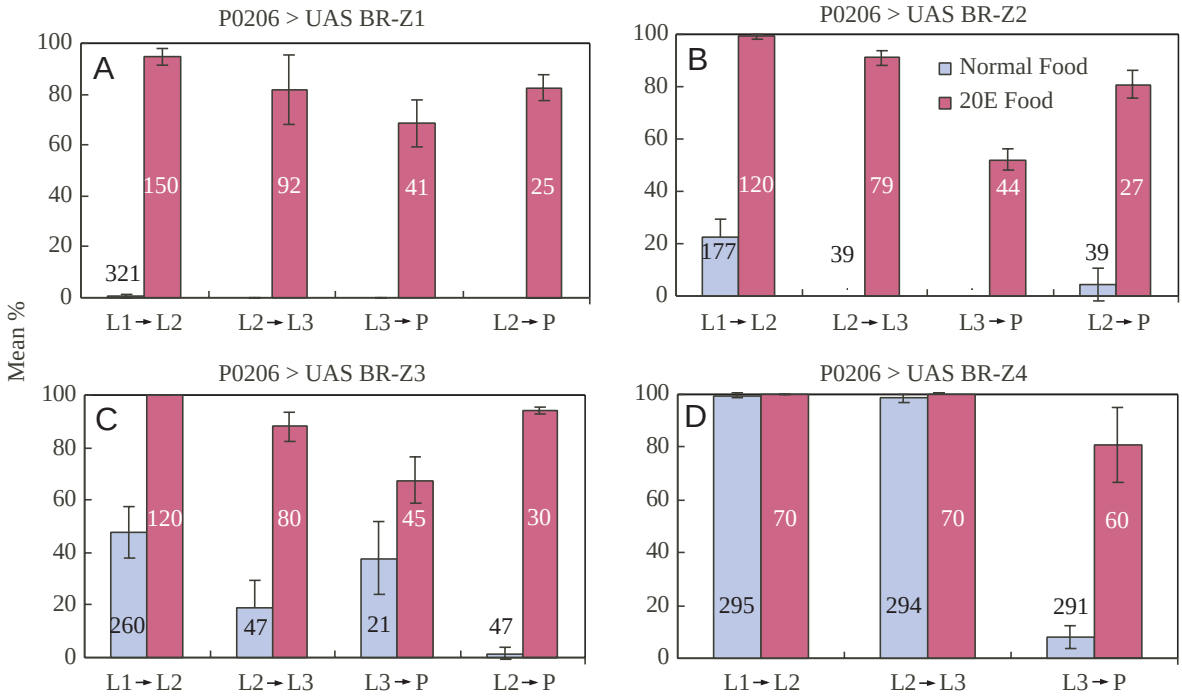


Fig. 5. The effect of ectopic expression of BR-Z isoforms in the prothoracic gland (PG) cells on *Drosophila* molts. (A–D) Larvae with ectopic expression of one of the BR isoforms in the PG cells under the control of the P0206 Gal4 driver were fed either standard diet or 20-hydroxyecdysone (20E) diet as described in Materials and methods. Each bar represents an average of at least three repetitions of the percentage of successful molting (mean ± S.D.). The total number of animals for each observation is shown in the bar.

Degeneration of the PG cells after ectopic expression of BR

Tonic expression of BR-Z3 in the ring gland under the control of the P0206 Gal4 driver caused degeneration of the PG portion of the ring gland by 2 days after ecdysis to the second instar (Fig. 6A, right). The CA were unaffected although the ectopic expression of BR-Z3 was throughout the ring gland. To visualize the process of degeneration of the PG portion of the ring gland, we used P0206 to drive each of the BR isoforms as well as green fluorescent protein (GFP) in the PG cells. Expressing GFP alone in the PG cells did not affect their growth during larval life (Fig. 6B, first row). By contrast, expression of BR-Z1 caused partial degeneration of the PG part of the ring gland by 24 h after hatching and disappearance by 35 h (Fig. 6B, second row). The BR-Z2 isoform caused severe degeneration by ~9 h after hatching, whereas degeneration induced by the BR-Z3 isoform was nearly complete at 48 h (Fig. 6B, third and fourth rows). In contrast to the other BR isoforms, BR-Z4 appeared to be rather ineffectual in causing PG cell degeneration (Fig. 6B, bottom row). Thus, the BR isoforms varied in their effectiveness in causing the degeneration of the PG cells.

Normal expression pattern of BR in the PG cells

To elucidate the role of BR proteins in the PG cells during metamorphosis, we determined the normal expression pattern of the four BR isoforms. BR-Z2 and -Z3 appear in the PG cells ~17 h after ecdysis to the third instar (89 h AEL) and remain at low levels through pupariation and the prepupal period (Fig. 7), showing little relationship with the changing ecdysteroid titer through this period. By contrast, BR-Z4 begins increasing slowly about 96 h AEL, then more rapidly at the time of wandering. BR-Z1 appears last, beginning at wandering, and also attains high levels by pupariation. Both of the latter isoforms apparently are responsive to 20E, which has increased to peak levels at this time (Riddiford, 1993).

Discussion

Except for neurons in the CNS (Zhou, 2000; B.Z., D. Williams, L.M.R. and J.W.T., unpublished), the ecdysteroid-induced transcription factor BR first appears in the final larval stage in preparation for metamorphosis. Premature expression of BR-Z3 in all tissues in the penultimate larval stage (L2) caused the suppression of the last larval molt and L2 prepupae were formed. These larvae could be induced to form pharate third instar larvae by feeding on 20E. These data are consistent with the hypothesis that the ectopic BR prevents the normal ecdysteroid rise required for the larval molt. As these larvae continued to feed and grow, they surpassed the threshold weight necessary for metamorphosis. The surpassing of this threshold allowed the premetamorphic program to be initiated, including the appearance of endogenous BR in a tissue-specific pattern. The GAL4–UAS experiments show that the target for this suppressive effect of BR is the prothoracic glands rather than the putative PTTH neurons.

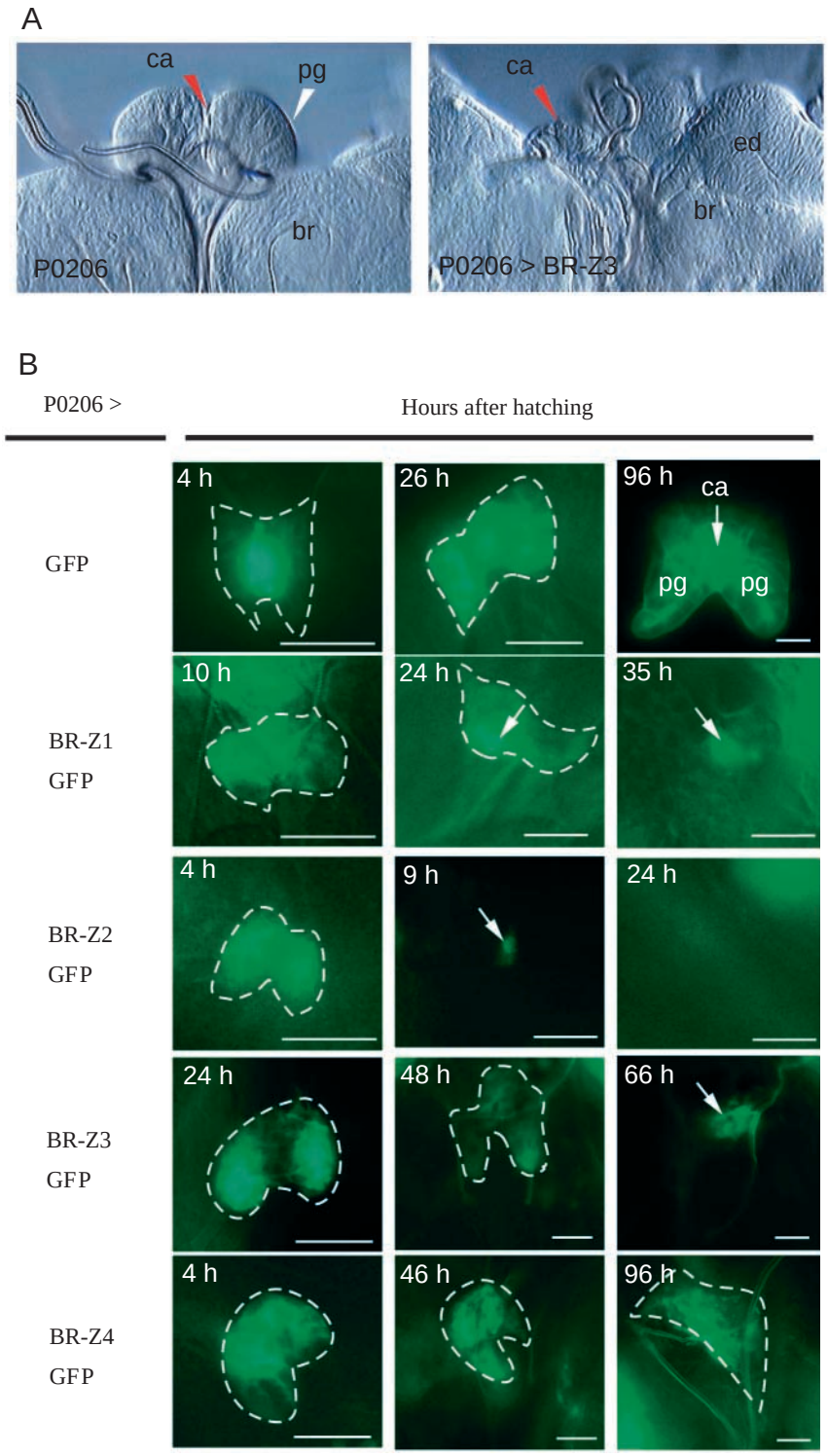


Fig. 6. Ectopic expression of Broad (BR) proteins in the prothoracic gland cells of the ring gland causes degeneration. (A) Expression of BR-Z3 in the ring gland driven by P0206 caused degeneration of the entire PG portion of the ring gland in a prolonged second instar larva (48 h after ecdysis into the second instar; right). Only part of the corpora allata (CA) remained intact (arrowhead). Compare to an intact ring gland of a second instar control larva (left). (B) The process of degeneration of the prothoracic gland (PG) cells was visualized by expressing both green fluorescent protein (GFP) and BR isoforms *via* P0206. The ring glands are outlined by broken lines, and the arrows point to the CA. Scale bar, 25 μm. Abbreviations: br, brain; ca, corpora allata; ed, eye disc; pg, prothoracic gland cells.

Critical weight and body size

For many insects, especially Lepidoptera, larval size is an important factor in regulating both larval molting and the onset of metamorphosis. Within a larval stage there is typically a minimal time that a larva must feed before it is competent to undertake another larval molt, originally called the 'period of indispensable nutrition' (Bounhiol, 1938; Nijhout, 1981). In addition, there is a size that the larva must attain before it can begin metamorphosis (the critical weight for metamorphosis; Nijhout, 1981). This size is thought to be largely independent of the number of larval molts required to reach it since larvae of the silkworm *Bombyx mori* undergo a series of extra larval molts at small sizes when fed ecdysone but eventually initiate metamorphosis at the appropriate size (Tanaka and Takeda, 1993). For second instar *Drosophila* larvae, the period of indispensable nutrition extends through the first 6.5 h of the instar, by which time the larva has attained a weight of 0.15 mg. By the time that the larva ecdyses to the third larval stage, its weight has increased to 0.52 mg.

The critical weight for metamorphosis is usually determined by starving larvae of various weights, then determining the timing of the subsequent onset of metamorphosis (Nijhout, 1975, 1981). Using this criterion, we obtained two values for this critical weight depending on whether the larva was forming a puparium in the third instar (0.8 mg) or in the second instar (0.45 mg). The 0.8 mg for the w^{1118} strain is slightly lower than the 0.9 mg found by Partridge et al. (1999) for their wild-type strain. It is important to stress that these weights indicate the condition of the larva when it initiated the metamorphic events but not necessarily its state when it becomes *competent* to metamorphose. The latter 'threshold weight' for metamorphosis was established by challenging larvae with 20E at various times. When this was done for L2 larvae whose molting had been suppressed by BR-Z3 misexpression, we found that the metamorphic threshold weight was 0.3 mg. We suggest that this threshold may be assessed independently of which instar the larva is in. First instar larvae cannot attain this

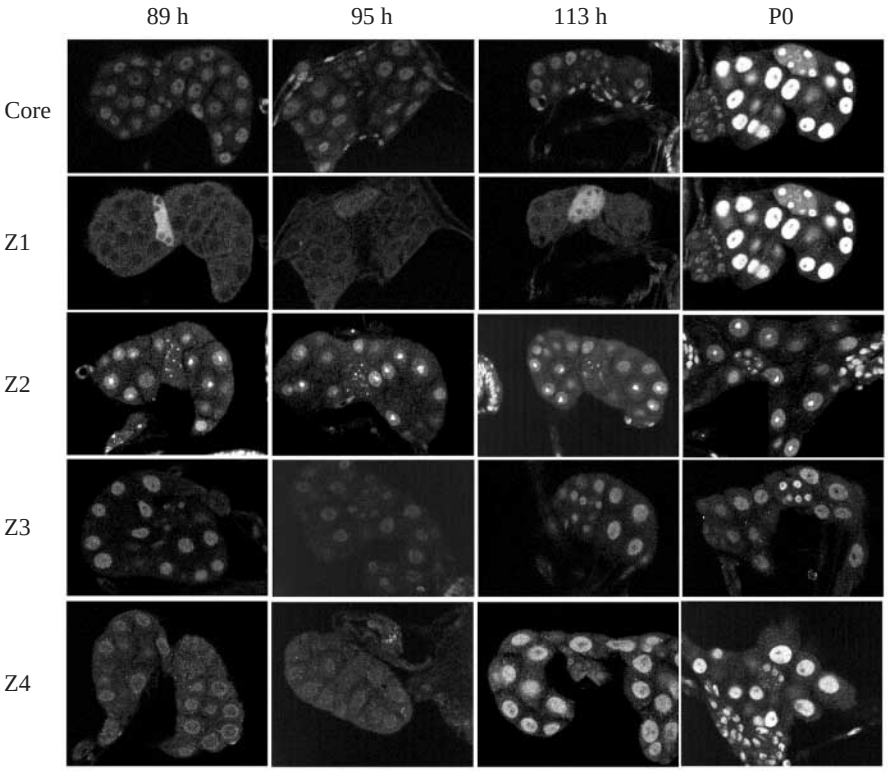


Fig. 7. Normal expression pattern of Broad (BR) proteins in the prothoracic gland (PG) cells. Third instar larvae or white puparia were dissected at designated times (hours after egg laying). Each ring gland was double stained with antibodies against BR core region and one of the BR isoforms as described in Materials and methods. Note that the anti-BR-Z2 antibody also nonspecifically stained the nucleoli.

threshold weight despite experimental manipulations that ensure prolonged feeding, probably because of cuticular restraints on their growth, and thus cannot pupariate. Normally growing second instar larvae traverse this threshold weight only after they have already initiated the molt to the third larval stage. Since the metamorphic threshold is crossed before the last instar is begun, higher Diptera such as *Drosophila* lack the molting plasticity seen in lepidopteran larvae. Consequently, in higher flies, a supernumerary fourth larval stage is never produced, irrespective of feeding conditions in the final larval stage. By interfering with ecdysteroid release in the second larval stage, however, larvae can attain the metamorphic threshold before being completely engaged in a larval molt and, hence, they can switch over into a precocious metamorphic program.

The formation of L2 prepupae is also reported for mutants of *dre4*, *iptr* and *E75A* (Sliter and Gilbert, 1992; Venkatesh and Hasan, 1997; Bialecki et al., 2002). These genes are known to affect the regulation of ecdysteroidogenesis in the PG cells. Thus, although these larvae may surpass the critical weight to initiate that larval molt, the onset of the molt is probably delayed because of the defective production of ecdysteroids. During the prolonged feeding, they have a chance to surpass the threshold weight for metamorphosis and begin premetamorphic changes. These larvae then regain the ability to produce some ecdysteroids, so they can form L2 prepupae. For example, young second instar larvae of a temperature-sensitive *dre4* mutant molt to the third instar after a temperature shift from 31°C to 22°C, while prolonged second instar larvae will form L2 prepupae after such a temperature shift (Sliter and Gilbert, 1992).

Misexpressed BR proteins block the rise of ecdysteroid titer for larval molting

We show here for the first time that *br* gene products can interfere with ecdysone synthesis and/or release by the larval PG cells. BR appears to have two types of effects on the PG cells: short-term effects on physiology of ecdysteroid synthesis and release, and longer-term effects that lead to eventual degeneration of the glands. We have concluded that the suppression of the larval molt caused by ectopic *br* expression is due to a suppression of ecdysone synthesis and release. This conclusion is based on rescue of larval molting in these larvae by supplying them with 20E in the diet. For the heat-shocked larvae, the 20E caused a larval molt but the animals were blocked at ecdysis. The latter effect, though, is presumably due to persistent BR effects in other larval tissues rather than the PG cells. The same 20E treatment resulted in normal larval molting and ecdysis of larvae that had larval molting suppressed by BR expression primarily in the ring gland.

The short-term action of BR-Z3 on the PG cells after heat shock induction clearly did not cause degeneration in the cells since they became functional once the induced BR disappeared so that L2 prepupae were formed. Similarly, a heat-shock-induced pulse of BR-Z1 in the early second instar prevented larvae from molting into the third instar, indicating that BR-Z1 also prevented the rise of the ecdysteroid titer. After this treatment, no L2 prepupae were formed. Instead, the larvae died ~50 h after the heat shock, indicating that some other essential tissues and/or organs had been adversely affected by the transient global expression of BR-Z1. Interestingly, neither BR-Z2 nor BR-Z4 had adverse effects on ecdysone biosynthesis or release in this paradigm, because pharate third instar larvae were formed. These however could not ecdyse, presumably due to other adverse effects of the presence of the particular BR isoform.

The manner by which BR-Z1 or -Z3 interferes with the functioning of the larval PG cells is unclear. During a larval molt there is no BR expression in PG cells but there is prominent BR expression during the time of the pupariation peak of ecdysteroid (Fig. 7). Moderate levels of BR-Z2 and -Z3 are evident in the gland early in the third instar while BR-

Z4 becomes prominent early in the ecdysteroid rise and BR-Z1 appears strongly at the pupariation peak of ecdysteroid. The late appearance of BR-Z1 would be consistent with its involvement in the suppression of ecdysone biosynthesis and release that has been noted at this time (Dai and Gilbert, 1991), and hence in the normal decrease of the ecdysteroid titer that occurs. Such an explanation is not possible for the effect of BR-Z3 since it is normally present throughout the metamorphic ecdysteroid peak. An alternative possibility is that BR-Z3 does not affect ecdysone biosynthesis directly but changes the sensitivity of the PG cells to growth-related factors. For example, factors from over-proliferating or regenerating imaginal discs are able to suppress the metamorphic ecdysone surges and prolong larval life (Simpson et al., 1980; Bryant and Simpson, 1984). Such factors would not be expected to interfere with larval molting that normally occurs as the discs are rapidly proliferating. Expression of BR-Z3 in the PG cells may be necessary for the glands to become responsive to these factors.

A recent study on overexpression of BR isoforms during the third instar further supports the suppressive function of BR on ecdysteroid biosynthesis (Kuchárová-Mahmood et al., 2002). Each of the BR isoforms was heat-shock induced before the rise of the small pulse of ecdysteroid titer that triggers the wandering behavior of larvae (Riddiford, 1993). When BR-Z1, -Z3 or -Z4 was ectopically expressed in 90–96-h-old feeding larvae, the feeding period was prolonged and pupariation delayed by 8–24 h. Ectopic expression of BR-Z2 during the mid-third instar, however, did not postpone pupariation, consistent with our observations that heat-shock-induced BR-Z2 in the second instar does not prevent larval molting. The BR-Z4 protein, whose ectopic expression can delay pupariation but is unable to prevent larval molting, may have some stage-specific activities.

In contrast to the effects of BR proteins, the ecdysteroid-induced orphan nuclear receptor E75A apparently promotes ecdysteroidogenesis (Bialecki et al., 2002). Approximately 55% of null *E75A* mutant larvae either arrest in the second instar or in the molt to the third instar, but the molt can be rescued by feeding 20E. An additional 20% form L2 puparia after a prolonged feeding period of about the same duration as we find after a pulse of BR-Z3 expression at 5 h after ecdysis. The ecdysteroid titer of these E75A null second instar larvae remains low for the first 36 h after ecdysis but must rise later in those undergoing L2 pupariation. Neither E75B nor E75C null mutants show any problems in larval molting or the onset of metamorphosis, indicating the lack of effect of these other isoforms on the ecdysteroid titer. Thus, E75A and BR-Z3 may act as positive and negative regulators, respectively, of ecdysteroid biosynthesis and release.

BR proteins cause the degeneration of the PG cells

In *Drosophila* during metamorphosis, the PG portion of the ring gland gradually degenerates. Its ecdysteroid biosynthetic activity (as measured by synthesis during a short term culture *in vitro*) dramatically decreases at pupariation followed by a more gradual decrease during the onset of adult development (Dai and Gilbert, 1991). At 24 h after pupariation (AP), lysosome-like structures invade the organelles in the PG cells and form giant autophagic vacuoles by 48 h AP. Only a few remnants of the PG cells are present at adult eclosion. We show here that BR normally appears in the PG cells ~17 h after ecdysis to the final larval instar, with the Z2 and the Z3 isoforms appearing first at low levels followed later by high levels of BR-Z1 and -Z4 at the time of pupariation. Since BR-Z1, -Z2 and -Z3 isoforms effectively cause the gradual degeneration of the PG cells, it is likely that the appearance of BR proteins at pupariation initiates the progressive involution of the gland.

Unlike the slow procedure of the degeneration of the PG cells, the larval salivary glands undergo rapid cell death within a few hours after pupation (Lee and Baehrecke, 2001). The larval salivary gland degenerates soon after pupal head eversion presumably in response to a pulse of ecdysteroid. Steroid-induced BR-Z1, βFTZ-F1, E74A and E93 are involved in the regulation of salivary gland programmed cell death (Jiang et al., 2000; Lee and Baehrecke, 2001; Lee et al., 2002). In the rbp^5 mutant, which lacks the BR-Z1 isoform, the transcription of the cell death genes *rpr* and *hid* is dramatically reduced (Jiang et al., 2000; Lee et al., 2002). In agreement with these studies, we found that in larvae misexpressing BR-Z1 in both the PG cells and the salivary glands, the salivary glands degenerated precociously in larvae rescued to the third instar by 20E treatment. By contrast, if these larvae were left in standard diet after hatching and stayed as permanent first instar, their PG cells degenerated within 35 h (Fig. 6) but their salivary glands remained intact for at least 72 h (X.Z. and L.M.R., unpublished). These observations suggest that in the PG cells, BR is sufficient to initiate and coordinate the program of degeneration even in the absence of 20E and molting. For the cell death of the salivary gland, by contrast, both BR and other 20E-induced factors are required.

We thank Dr Cynthia Bayer for the transgenic *hs-BR* lines, Drs Thomas Siegmund and Gunther Korge for the Feb 204 and Feb 211 lines, Dr Wilfried Janning for the P0206 and P0163 lines, Dr Gregory Guild for the BR core antibody and Dr Jean-Antoine Lepesant for BR isoform-specific antibodies. Fly stocks were maintained by Nicholas Jansson. This study was supported by NIH GM60122 and NS29971.

References

Baehrecke, E. H. (2000). Steroid regulation of programmed cell death during *Drosophila* development. *Cell Death Differ.* **7**, 1057-1062.

Bainbridge, S. P. and Bownes, M. (1981). Staging the metamorphosis of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* **66**, 57-80.

Bayer, C. A., Holley, B. and Fristrom, J. W. (1996). A switch in *Broad-Complex* zinc-finger isoform expression is regulated posttranscriptionally during the metamorphosis of *Drosophila* imaginal discs. *Dev. Biol.* **177**, 1-14.

Bayer, C. A., von Kalm, L. and Fristrom, J. W. (1997). Relationships between protein isoforms and genetic functions demonstrate functional redundancy at the *Broad-Complex* during *Drosophila* metamorphosis. *Dev. Biol.* **187**, 267-282.

Belyaeva, E. S., Aizenzon, M. G., Semeshin, V. F., Kiss, I. I., Koczka, K., Baritcheva, E. M., Gorelova, T. D. and Zhimulev, I. F. (1980). Cytogenetic analysis of the 2B3-4–2B11 region of the X-chromosome of *Drosophila melanogaster*. I. Cytology of the region and mutant complementation groups. *Chromosoma* **81**, 281-306.

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W. A. and Thummel, C. S. (2002). Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. *Dev. Cell* **3**, 209-220.

Bodenstein, D. (1965). The postembryonic development of *Drosophila*. In *Biology of* Drosophila. (ed. M. Demerec), pp. 275-367. New York: Hafner Publishing Co.

Bounhiol, J. J. (1938). Récherches experimentales sur le determinisme de la metamorphose chez les Lepidopterès. *Biol. Bull.* **Suppl. 24**, 1-199.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brennan, C. A., Li, T. R., Bender, M., Hsiung, F. and Moses, K. (2001). *Broad-complex*, but not *ecdysone receptor*, is required for progression of the morphogenetic furrow in the *Drosophila* eye. *Development* **128**, 1-11.

Bryant, P. J. and Simpson, P. (1984). Intrinsic and extrinsic control of growth in developing organs. *Q. Rev. Biol.* **59**, 387-415.

Cakouros, D., Daish, T., Martin, D., Baehrecke, E. H. and Kumar, S. (2002). Ecdysone-induced expression of the caspase DRONC during hormone-dependent programmed cell death in *Drosophila* is regulated by Broad-Complex. *J. Cell Biol.* **157**, 985-995.

Crossgrove, K., Bayer, C. A., Fristrom, J. W. and Guild, G. M. (1996). The *Drosophila Broad-Complex* early gene directly regulates late gene transcription during the ecdysone-induced puffing cascade. *Dev. Biol.* **180**, 745-758.

Dai, J. D. and Gilbert, L. I. (1991). Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of *Drosophila melanogaster*: a cytophysiological analysis of the ring gland. *Dev. Biol.* **144**, 309-326.

DiBello, P. R., Withers, D. A., Bayer, C., Fristrom, J. W. and Guild, G. M. (1991). The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. *Genetics* **129**, 385-397.

Emery, I. F., Bedian, V. and Guild, G. M. (1994). Differential expression of *Broad-Complex* transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. *Development* **120**, 3275-3287.

Ghbeish, N., Tsai, C. C., Schubiger, M., Zhou, J. Y., Evans, R. M. and McKeown, M. (2001). The dual role of ultraspiracle, the *Drosophila* retinoid X receptor, in the ecdysone response. *Proc. Natl. Acad. Sci. USA* **98**, 3867-3872.

Gilbert, L. I., Rybczynski, R. and Warren, J. T. (2002). Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol.* **47**, 883-916.

Huet, F., Ruiz, C. and Richards, G. (1993). Puffs and PCR: the in vivo dynamics of early gene expression during ecdysone responses in *Drosophila*. *Development* **118**, 613-627.

Jiang, C., Lamblin, A. F., Steller, H. and Thummel, C. S. (2000). A steroid-triggered transcriptional hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. *Mol. Cell.* **5**, 445-455.

Kiriishi, S., Rountree, D. B., Sakurai, S. and Gilbert, L. I. (1990). Prothoracic gland synthesis of 3-dehydroecdysone and its hemolymph 3β-reductase mediated conversion to ecdysone in representative insects. *Experientia* **46**, 716-721.

Kiss, I., Beaton, A. H., Tardiff, J., Fristrom, D. and Fristrom, J. W. (1988). Interactions and developmental effects of mutations in the *Broad-Complex* of *Drosophila melanogaster*. *Genetics* **118**, 247-259.

Kiss, I., Bencze, G., Fodor, G., Szabad, J. and Fristrom, J. W. (1976). Prepupal–larval mosaics in *Drosophila melanogaster*. *Nature* **262**, 136-138.

Kuchárová-Mahmood, S., Raska, I., Mechler, B. M. and Farkas, R. (2002). Temporal regulation of *Drosophila* salivary gland degeneration by the *Broad-Complex* transcription factors. *J. Struct. Biol.* **140**, 67-78.

Lee, C. Y. and Baehrecke, E. H. (2001). Steroid regulation of autophagic programmed cell death during development. *Development* **128**, 1443-1453.

Lee, C. Y., Simon, C. R., Woodard, C. T. and Baehrecke, E. H. (2002). Genetic mechanism for the stage- and tissue-specific regulation of steroid triggered programmed cell death in *Drosophila*. *Dev. Biol.* **252**, 138-148.

Mugat, B., Brodu, V., Kejzlarova-Lepesant, J., Antoniewski, C., Bayer, C. A., Fristrom, J. W. and Lepesant, J.-A. (2000). Dynamic expression of *Broad-Complex* isoforms mediates temporal control of an ecdysteroid target gene at the onset of *Drosophila* metamorphosis. *Dev. Biol.* **227**, 104-117.

Nijhout, H. F. (1975). A threshold size for metamorphosis in the tobacco hornworm, *Manduca sexta*. *Biol. Bull.* **149**, 214-225.

Nijhout, H. F. (1981). Physiological control of molting in insects. *Am. Zool.* **21**, 631-640.

Nijhout, H. F. (1994). *Insect Hormones*. Princeton: Princeton University Press.

Partridge, L., Langelan, R., Fowler, K., Zwaan, B. and French, V. (1999). Correlated responses to selection on body size in *Drosophila melanogaster*. *Genet. Res.* **74**, 43-54.

Riddiford, L. M. (1993). Hormones and *Drosophila* development. In *The Development of* Drosophila melanogaster (ed. M. Bate and A. Martinez-Arias), pp. 899-939. Plainview: Cold Spring Harbor Laboratory Press.

Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.

Russell, S. R. H. and Ashburner, M. (1996). Ecdysone-regulated chromosome puffing in *Drosophila melanogaster*. In *Metamorphosis: Post-embryonic Reprogramming of Gene Expression in Amphibian and Insect Cells* (ed. L. I. Gilbert, B. Atkinson and J. R. Tata), pp. 109-144. San Diego: Academic Press.

Siegmund, T. and Korge, G. (2001). Innervation of the ring gland of *Drosophila melanogaster*. *J. Comp. Neurol.* **431**, 481-491.

Simpson, P., Berreur, P. and Berreur-Bonnenfant, J. (1980). The initiation of pupariation in *Drosophila*: dependence on growth of the imaginal discs. *J. Embryol. Exp. Morph.* **57**, 155-165.

Sliter, T. J. and Gilbert, L. I. (1992). Developmental arrest and ecdysteroid deficiency resulting from mutations at the *dre4* locus of *Drosophila*. *Genetics* **130**, 555-568.

Sullivan, W., Ashburner, M. and Hawley, R. S. (2000). Drosophila *Protocols*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Tanaka, Y. and Takeda, S. (1993). Ultranumerary larval ecdyses of the silkworm, *Bombyx mori* induced by ecdysone. *Naturwissenschaften* **80**, 131-132.

Thummel, C. S. (1996). Files on steroids – *Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends. Genet.* **12**, 306-310.

Thummel, C. S. (2002). Ecdysone-regulated puff genes 2000. *Insect Biochem. Mol. Biol.* **32**, 113-120.

Truman, J. W. (1972). Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. Exp. Biol.* **57**, 805-820.

Venkatesh, K. and Hasan, G. (1997). Disruption of the IP3 receptor gene of *Drosophila* affects larval metamorphosis and ecdysone release. *Curr. Biol.* **7**, 500-509.

von Kalm, L., Crossgrove, K., Von Seggeern, D., Guild, G. M. and Beckendorf, S. K. (1994). The *Broad-Complex* directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. *EMBO J.* **13**, 3505-3516.

Zhou, B. (2000). *E75* and *Broad-complex*: two JH-regulated genes in the ecdysone signaling pathway. *Ph.D.Thesis*. University of Washington.

Zhou, B., Hiruma, K., Shinoda, T. and Riddiford, L. M. (1998). Juvenile hormone prevents ecdysteroid-induced expression of Broad Complex RNAs in the epidermis of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* **203**, 233-244.

Zhou, B. and Riddiford, L. M. (2001). Hormonal regulation and patterning of the *Broad-Complex* in the epidermis and wing discs of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* **231**, 125-137.

Zhou, X. and Riddiford, L. M. (2002). Broad specifies pupal development and mediates the 'status quo' action of juvenile hormone on the pupal–adult transformation in *Drosophila* and *Manduca*. *Development* **129**, 2259-2269.

Zollman, S., Godt, D., Prive, G. G., Couderc, J. L. and Laski, F. A. (1994). The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **91**, 10717-10721.