RESEARCH ARTICLE

Forward and feedback regulation of cyclic steroid production in Drosophila melanogaster

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ABSTRACT

In most animals, steroid hormones are crucial regulators of physiology and developmental life transitions. Steroid synthesis depends on extrinsic parameters and autoregulatory processes to fine-tune the dynamics of hormone production. In Drosophila, transient increases of the steroid prohormone ecdysone, produced at each larval stage, are necessary to trigger moulting and metamorphosis. Binding of the active ecdysone (20-hydroxyecdysone) to its receptor (EcR) is followed by the sequential expression of the nuclear receptors E75, DHR3 and βFtz-f1, representing a model for steroid hormone signalling. Here, we have combined genetic and imaging approaches to investigate the precise role of this signalling cascade within the prothoracic gland (PG), where ecdysone synthesis takes place. We show that these receptors operate through an apparent unconventional hierarchy in the PG to control ecdysone biosynthesis. At metamorphosis onset, DHR3 emerges as the downstream component that represses steroidogenic enzymes and requires an early effect of EcR for this repression. To avoid premature repression of steroidogenesis, E75 counteracts DHR3 activity, whereas EcR and βFtz-f1 act early in development through a forward process to moderate DHR3 levels. Our findings suggest that within the steroidogenic tissue, a given 20-hydroxyecdysone peak induces autoregulatory processes to sharpen ecdysone production and to confer competence for ecdysoid biosynthesis at the next developmental phase, providing novel insights into steroid hormone kinetics.

KEY WORDS: Ecdysone, Prothoracic gland, Nuclear receptor, DHR3, Drosophila

INTRODUCTION

Animal development is controlled by a wide variety of messengers, including steroids that trigger developmental life transitions. In humans, the puberty phase is accompanied by an increase in steroid hormone production within the somatic cells of the gonads (Martos-Moreno et al., 2010). Although steroid production by the gonads has been shown to depend on positive- and negative-feedback loops (Navarro and Tena-Sempere, 2012), the molecular mechanisms that coordinate steroidogenesis with other intrinsic and extrinsic parameters remain poorly understood. Drosophila genetics has provided a powerful tool for deciphering how steroid production is controlled and subsequently signalled in target tissues (Ou and King-Jones, 2013; Rewitz et al., 2013; Yamanaka et al., 2013). In Drosophila, the steroid prohormone ecdysone is produced within the prothoracic gland (PG) cells, which are part of the ring gland (Bodenstein, 1994). Ecdysone is converted into the active hormone 20-hydroxyecdysone (20E) in the peripheral tissues (Petryk et al., 2003) to trigger moulting and metamorphosis (Riddiford et al., 2003). These processes require a precise kinetics of ecdysone production (Woodard et al., 1994).

Earlier studies on crustaceans and lepidopterans suggested that ecdysteroids may control their own biosynthesis (Beydon and Lafont, 1983; Dell et al., 1999; Sakurai and Williams, 1989) – a notion further supported by Drosophila studies. Binding of 20E to its receptor, EcR (Riddiford et al., 2000), induces a set of primary-response genes. These genes include E75 (Eip75B – FlyBase), which encodes three distinct nuclear receptor (NR) isoforms: E75A, B and C (Segraves et al., 2003) to trigger moulting and metamorphosis (Riddiford et al., 2003). In this process, βFtz-f1 is another NR induced by ecdysone (Hiruma and Riddiford, 2004; Horner et al., 1995), which together with E75B regulates transcription of the downstream NR βFtz-f1, the β-isofrom encoded by the ftz-f1 gene (Palanker et al., 2006; Reinking et al., 2005; White et al., 1997). The idea of steroidogenesis autoregulation in Drosophila arises from phenotypic analyses showing that E75A mutants fail to produce ecdysteroids during the second larval stage (Bialecki et al., 2002). Furthermore, our past study has shown that the steroidogenic enzymes Phantom (Phm) and Disembodied (Dib) are not detected in ftz-f1 mutant PG cells (Parvy et al., 2005), suggesting that the NRs induced by 20E signalling also act in PG cells to control steroidogenesis.

Here, we have investigated how the 20E-responsive NR cascade controls ecdysone biosynthesis within the steroidogenic tissue. Our findings indicate that 20E signalling does not directly activate ecdysone biosynthesis, but rather acts to potentiate and to narrow the cyclic production of the steroid hormone. Instead of transcriptional activation, we observed that DHR3 acts as a repressor of ecdysone production and that it downregulates expression of the steroidogenic enzymes at the onset of metamorphosis. In this process, βFtz-f1 and EcR moderate DHR3 levels. In addition, EcR is required earlier in development for the DHR3-mediated repression, whereas E75 counteracts the DHR3-inhibiting activity.

RESULTS

NRs mediating 20E signal are expressed in the PG cells

Based on an autoregulatory model, the PG cells should respond to 20E and thus should express the NRs induced by 20E stimulation. To investigate the expression of EcR, E75, DHR3 and βFtz-f1, we carried out ring gland immunostaining at stages corresponding to the major ecdysteroid peaks (Fig. 1A): mid/late L1 (12-20 h after hatching), mid/late L2 (12-20 h past the L1-L2 moult), wandering L3 (40-48 h past the L2-L3 moult), and early prepupa (0-6 h past pupariation) (Fig. 1A,B). EcR (Fig. 1D,H,L,P), DHR3 (Fig. 1F,J,N,R) and βFtz-f1 (Fig. 1G,K,O,S) were present in the nuclei of PG cells at all stages.
Nuclear E75 was detected in the PG cells of L1, L2 and wandering L3 larvae but not in prepupae (Fig. 1E,I,M,Q).

To evaluate the precise kinetics of 20E signalling in the PG, expression of EcR, E75, DHR3 and βFtz-f1 was analysed every 4 h from the early L2 to late L3 stages (Fig. 1C and supplementary material Figs S1-S5). EcR expression increased from early to mid-L2 (supplementary material Fig. S1A-E) and then decreased to reach a minimum in early L3 larvae (supplementary material Fig. S1F-H). During the L3 stage, EcR expression increased to a maximum in 40 h L3 larvae and then decreased at later stages (supplementary material Fig. S1I-T). The E75A (supplementary material Fig. S2) and E75B (supplementary material Fig. S3) isoforms were analysed with specific antibodies. E75A protein was weakly expressed in early L2 larvae (supplementary material Fig. S2A-C), reached a maximum at 12-16 h (supplementary material Fig. S2D,E) and then decreased to reach another minimum in late L2 larvae (supplementary material Fig. S2F,G). During L3 stage (supplementary material Fig. S2H-T), peaks of expression were observed at 8 h (supplementary material Fig. S2J), 24 h (supplementary material Fig. S2N) and 36-44 h (supplementary material Fig. S2Q-S), and then E75A levels dropped at late L3 stage (supplementary material Fig. S2T). E75B (supplementary material Fig. S3A-T) is weakly detected only in the cytoplasm. Low levels of nuclear DHR3 were detected at 4-8 h during L2 stage (supplementary material Fig. S4A-C). A peak of expression was observed at 12-16 h then DHR3 disappeared at late L2 stage (supplementary material Fig. S4D-G). During L3 stage, three peaks of nuclear DHR3 were detected at 8 h, 24-28 h and 44-48 h (supplementary material Fig. S4H-T). βFtz-f1 was highly expressed in early L2 (supplementary material Fig. S5A-C), became barely detectable at the mid-L2 stage (supplementary material Fig. S5D-G), and then E75A levels dropped at late L3 stage (supplementary material Fig. S5H-T). βFtz-f1 expression was maintained in early L3 larvae (supplementary material Fig. S5J) and progressively decreased to become barely detectable past the mid-L3 stage (supplementary material Fig. S5K-P). At late L3 stage, βFtz-f1 expression slightly increased (supplementary material Fig. S5R-T). The observation that these four NRs are expressed in the PG at each larval stage suggests that this tissue responds to 20E.
NR misexpression in the PG provokes developmental arrests rescued by 20E

To determine whether EcR, E75, DHR3 and βFtz-f1 are required for ecdysone production in the PG, we used the Gal4/UAS system to knock down each NR using specific UAS-RNAi transgenes (hereafter referred to as NRx-RNAi). To monitor gene silencing, we examined both transcript and protein levels in RNAi-expressing flies. Transcripts were quantified by real-time quantitative PCR using a ubiquitous tub-gal4 driver combined with a ubiquitous thermosensitive form of the Gal4 inhibitor, Gal80ts (tub-gal80ts) that blocks Gal4 activity at 21°C but not at 29°C, thereby allowing RNAi expression after the temperature shift. Each NR-RNAi was ubiquitously induced during the late L3 stage and transcript levels were measured every hour during the prepupal stage. At each time point analysed, the RNAi dropped the total amount of the corresponding NR transcript (supplementary material Fig. S6A-D). Protein levels were analysed by immunostaining in PG containing clonal cells generated by random recombination (flip-out) to express each RNAi (supplementary material Fig. S6A-D'). In this setting, each RNAi induced almost complete protein extinction (supplementary material Fig. S6A-D').

Next, we induced various UAS transgenes with the phm-gal4 driver, which is active from late embryogenesis in the steroidogenic tissue. Considering that ecdysteroids induce moulting and metamorphosis, we anticipated that knocking down an NR required for steroid production would lead to developmental arrest. We carried out experiments at 25°C and 29°C, as higher temperatures increase Gal4/UAS efficacy. This silencing provoked differential developmental arrests depending on the targeted NR. Knockdown of EcR at 29°C provoked developmental arrest at the end of the L3 stage; however, at 25°C, several escapers underwent metamorphosis (Fig. 2A,B). The L3-arrested larvae kept foraging and continued to grow for several more days (supplementary material Fig. S7F-H). Consistently, directing an EcR-dominant Fig. 2. NR misregulations provoke developmental arrests suppressed by ecdysone feeding. (A,B) Quantification of the phenotypes induced by misexpression of NRs using the phm-gal4 driver. RNAi (NRx-Ri) and overexpressing (UAS-NRx) lines are indicated at the bottom. The percentages of the various phenotypes are represented as proportional bars corresponding to developmental arrest at the late L2 (red), late L3 (orange) and pupal (yellow) stages, or to adult survival (green). The experiments were performed at 25°C (A) or 29°C (B). (C) Ring glands of late L2 (top) or late L3 (bottom) larvae expressing various transgenes together with a UAS-mCD8::GFP, using the phm-gal4 driver. Top panel from left to right: control (i), E75-RNAi (ii), ftz-f1-RNAi (iii), UAS-DHR3 (iv). Bottom panel from left to right: control (i'), EcR-RNAi (ii'), UAS-E75 (iii'), DHR3-RNAi (iv'). Scale bars: 20 μm. (D) The developmental arrests at late L2 (top) or L3 (bottom) stages induced by NR misregulation could be rescued on ecdysone-containing medium (20E) but not on control medium (EtOH). Rescue experiments were performed at 29°C. Phenotypes are indicated as in A,B. DHR3-RNAi shown (20157).
negative form with the phm-gal4 driver produced the same phenotype at 29°C (data not shown). At both temperatures, knockdown of E75 provoked a developmental arrest at the end of the L2 stage, although at 25°C, few larvae underwent pupariation (Fig. 2A,B and supplementary material Fig. S7K,M). Knockdown of DHR3 did not provoke developmental arrest at 25°C, whereas at 29°C it resulted in incomplete developmental arrest at the end of the L3 stage (Fig. 2A,B and supplementary material Fig. S7K,M). Expression of ftz-f1-RNAi in the PG led to developmental arrest at the late L2 stage either partial or total, when performed at 25°C or 29°C, respectively (Fig. 2A,B). The arrested L2 larvae continued to grow for up to 15 days and eventually died as giant L2 (supplementary material Fig. S7A,D,E). Using the same driver, overexpression of either DHR3 or E75A provoked developmental arrest at the late L2 and L3 stages, respectively (Fig. 2A,B and supplementary material Fig. S7F,I,J). Altogether, these findings indicate that these NRs differentially contributed to controlling the moulting transitions and suggest that in PG cells 20E signalling does not simply activate ecdysone biogenesis through the downstream NR βFtz-f1.

To determine whether expression of the above-mentioned RNAi-induced cellular defect in the PG cells may be responsible for the different phenotypes, the NR-RN4i were co-expressed with a UAS-mCD8::GFP using the phm-gal4 driver. Observations of the ring glands did not reveal any visible cellular defect of the PG cells (Fig. 2C), indicating that the developmental arrests are not due to cell death but rather result from a dysfunction of steroidogenesis. To investigate whether ecdysteroid deficiency was responsible for the developmental arrests, the feeding medium was supplemented with 20E. In the case of L2 arrest, feeding 20E rescued all the larvae expressing either ftz-f1-RNAi or E75-RNAi and 70% of the larvae overexpressing DHR3 (Fig. 2D). For late L3 arrest, feeding 20E rescued almost half of the larvae expressing either EcR-RNAi or UAS-E75A and the quasi-totality of the larvae expressing DHR3-RNAi (Fig. 2D). This 20E-dependent rescue demonstrates that the developmental arrests following NR misregulation in the PG cells are due to the lack of ecdysteroid at the L2 or L3 stages, showing that 20E controls its own biosynthesis.

**NRs act through an unconventional hierarchy in the PG**

To decipher the molecular mechanisms of 20E autoregulation, we investigated whether the canonical hierarchy of NRs is conserved in the PG. First, we analysed the expression of DHR3 and βFtz-f1 in flip-out clones expressing EcR-RNAi. In this setting, DHR3 levels were faintly decreased at L2 stage but strongly increased at late L3 stage (Fig. 3A-F). βFtz-f1 levels were decreased in EcR-RNAi flip-out cells of either late L2 or early L3 larvae (Fig. 3G-L). These findings indicate that EcR is required for the accurate dynamic expression of DHR3 and βFtz-f1.

These observations prompted us to investigate whether DHR3 controls βFtz-f1 expression in PG cells, as reported for other tissues (Kageyama et al., 1997; Lam et al., 1997; White et al., 1997). Therefore, we analysed βFtz-f1 levels in ring glands containing homozygous DHR3 mutant cells. We induced random mitotic recombination during embryogenesis and monitored βFtz-f1 expression at the mid-L2 (8-16 h past the L1/L2 moult), late L2 (16-24 h past the L1/L2 moult), early L3 (8-24 h past the L2/L3 moult), mid-L3 (20 to 28 h past L2/L3 moult) and late L3 (30 to 48 h past L2/L3 moult) stages. Surprisingly, the consequence of the DHR3 mutation on βFtz-f1 protein expression varied with the developmental stage. At mid-L2 or late L3 stages, when DHR3 is normally expressed and βFtz-f1 barely detected, there was no effect on βFtz-f1 expression in DHR3 mutant cells (Fig. 3M,N,Q and data not shown). At late L2 stage, when βFtz-f1 is strongly expressed, most of the DHR3 homozygous mutant cells exhibited a severe drop in βFtz-f1 levels compared with control cells (Fig. 3O-Q). By contrast, at later stages, when βFtz-f1 expression decreased in control cells, far fewer DHR3 mutant cells exhibited a visible drop in βFtz-f1 levels.
βFtz-f1 expression levels (Fig. 3Q and data not shown). In summary, although DHR3 is required for the highest βFtz-f1 expression levels before the L2/L3 moulting transition, it is dispensable at later stages, suggesting that βFtz-f1 can eventually be induced through a DHR3-independent mechanism.

Lowering DHR3 can rescue L2 developmental arrests
To investigate the hierarchy of E75, βFtz-f1 and DHR3 in PG cells, we performed genetic interactions using UAS transgene combinations. To normalize the number of UAS promoters in the combination settings, we buffered Gal4 activity with a UAS-GFP when driving only a single UAS transgene. In addition, to verify RNAi efficacy, knockdown has been checked in flip-out clones expressing RNAi combinations (supplementary material Fig. S8). Immunostaining confirmed that each RNAi line was efficient to drop protein levels of the targeted NR (supplementary material Fig. S8A,B,E-O), whereas co-expression of ftz-f1-RNAi and UAS-E75A did not impede E75A overexpression (supplementary material Fig. S8C,D). The genetic interactions were performed at 25°C using the phm-gal4 driver. E75-RNAi led to developmental arrest at L2 (74%) or pupal (26%) stages, whereas DHR3-RNAi did not provoke a visible phenotype (Fig. 4A). Expressing E75-RNAi and DHR3-RNAi together provoked an intermediate phenotype: 40% of L2 larvae, 6% of pupae and 54% as adults (Fig. 4A). These observations indicate that DHR3 downregulation can partially rescue the phenotype induced by E75 knockdown.

The knockdown of βFtz-f1 provoked developmental arrest: at the late L2 (20%) or late L3 (80%) stages (Fig. 4A). This phenotype was partially rescued by co-expression of ftz-f1-RNAi with either UAS-E75A or DHR3-RNAi (Fig. 4A). In these genetic combinations, no arrested L2 larvae were observed, and some giant L3 (25%) and many pupae (75%) formed, although the pupae never emerged as adult. This phenotypic suppression indicates that lowering DHR3 or increasing E75 expressions in PG cells counteracts βFtz-f1 knockdown.

Consistently, we observed that flip-out PG cells of L3 larvae expressing ftz-f1-RNAi exhibited a moderate increase of nuclear DHR3 levels (Fig. 4B,C), a phenotype that could also be observed in homozygous ftz-f1<sup>1177</sup> PG mutant cells at late L3 stage but barely at L2 stage (Fig. 4D-G). This increased nuclear DHR3 disappeared when co-expressing ftz-f1-RNAi and DHR3-RNAi (Fig. 4H,I). Importantly, the increase in DHR3 levels in ftz-f1-RNAi flip-out cells was observed in late L3 larvae while βFtz-f1 was weakly expressed in the PG (Fig. 4F,G). Considering that E75 has been described to counteract DHR3 activity (Palanker et al., 2006; Reinking et al., 2005) and that DHR3 overexpression provokes developmental arrest at late L2 stage, the developmental arrest due to E75-RNAi or ftz-f1-RNAi could be a consequence of higher DHR3 activity.

DHR3 represses steroidogenic enzyme expression
Given that βFtz-f1 is required for expression of the steroidogenic enzymes Phm and Dib in L3 larvae (Parvy et al., 2005), we investigated the influence of DHR3 on steroidogenic enzyme expression in late L3 larvae. Consistently, Phm levels decreased in flip-out PG cells expressing ftz-f1-RNAi (Fig. 5A,B), a phenotype that we also observed in E75-RNAi flip-out cells (Fig. 5C,D). As lowering DHR3 counteracts the developmental arrests induced by ftz-f1- and E75-RNAi, we hypothesized that downregulation of Phm expression might directly depend on DHR3-mediated repression. Immunostaining revealed that PG cells overexpressing the UAS-DHR3 transgene exhibited a dramatic drop in Phm, Dib and Sad expression in late L3 larvae (Fig. 5E,F; supplementary material Fig. S9A-D). Conversely, Phm and Dib expression significantly increased in homozygous DHR3 mutant cells (Fig. 5G,H; supplementary material Fig. S9E,F) indicating that, at the late L3 stage, DHR3 represses steroidogenic enzymes. However, the expression of Phm did not appear to be increased in DHR3-RNAi flip-out clones (Fig. 5I,J) possibly due to incomplete DHR3 knockdown. Nevertheless, DHR3-RNAi was able to restore Phm

Fig. 4. Genetic hierarchy of NRs in the ring gland.
(A) Quantification of the phenotypes obtained by inducing various transgene combinations in the ring gland. The phm-gal4 driver has been used to induce combinations of two of these transgenes: UAS-GFP, DHR3-RNAi (20157 when used alone), ftz-f1-RNAi, E75-RNAi and UAS-E75A. Phenotype quantification is represented as in Fig. 2B (n: number of animals analysed). (B-I) DHR3 staining (C,E,G,I) of ring glands containing GFP-labelled flip-out clones (B,H, arrowheads in C,I) or homozygote ftz-f1<sup>1177</sup> mutant clones marked by the lack of GFP (dashed line in D,F, arrowheads in E,G). DHR3 levels in ftz-f1-RNAi flip-out clones of late L3 larvae (B,C), and in ftz-f1<sup>1177</sup> mutant clones of late L2 (D,E) or late L3 (F,G) larvae. The increased in nuclear DHR3 levels is lost in flip-out clones co-expressing DHR3-RNAi with ftz-f1-RNAi (H,I). Scale bars: 20 μm. For clonal analysis the stages of heat shock and dissection are indicated as in Fig. 3.
levels when co-expressed with either ftz-f1-RNAi or E75-RNAi (Fig. 5K-N). The Phm levels were also restored when coexpressing UAS-E75 with ftz-f1-RNAi (supplementary material Fig. S9G,H). Downregulation of Phm may be responsible for the developmental arrest induced by DHR3 overexpression or activity in late L3 larvae. By contrast, we could not detect repression of Phm in flip-out PG cells of L2 or early L3 larvae expressing either E75-RNAi, ftz-f1-RNAi or UAS-DHR3 (supplementary material Fig. S9I-P) indicating that DHR3 can induce an ecdysone-dependent developmental arrest at the L2/L3 transition through a Phm-independent process. Taken together, these findings indicate that neither βFtz-f1 nor E75 directly activate the expression of steroidogenic enzymes, but rather maintain Phm levels by counteracting DHR3-mediated repression.

As DHR3 expression increased in EcR-RNAi PG cells in late L3 larvae (Fig. 3D-F), the expression of Phm might also be downregulated in these cells. Surprisingly, Phm levels remained unaffected in EcR-RNAi flip-out clones of late L3 larvae (Fig. 5O-Q), albeit with higher DHR3 levels. Consistently, Phm expression levels were not affected in flip-out clones expressing the dominant negative form of EcR (supplementary material Fig. S9T,U). As EcR has been shown to physically interact with DHR3 (White et al., 1997), we hypothesized that EcR could be required for DHR3-mediated repression of Phm. Accordingly, the drop in Phm levels was not observed in flip-out clones expressing EcR-RNAi (Fig. 5R,S), indicating that DHR3 requires EcR to repress Phm expression. In addition, repression of Phm was no longer observed in either E75-RNAi or ftz-f1-RNAi flip-out clones of late L3 larvae that co-express EcR-RNAi (Fig. 5T-W). Hence, DHR3 emerges as a crucial negative regulator of steroidogenic enzyme expression and requires EcR in this process.

Fig. 5. DHR3 suppresses Phm protein levels. Nuclear (A,C,E,I,K,M,O,R,T,V), Phm (B,D,F,J,L,N,P,S,U,W) and EcR (Q) staining in ring gland containing flip-out clones labelled by GFP (A,C,E,I,K,M,O,R,T,V, arrowheads in B, D,F,J,L,N,P,Q,S,U,W) or DHR3G60S mutant clones labelled by the absence of GFP (dashed line in G, arrowheads in H). Phm protein levels in flip-out clones expressing ftz-f1-RNAi (A,B), E75-RNAi (C,D), UAS-DHR3 (E,F), in DHR3G60S mutant clones (G,H), and in DHR3-RNAi (20157) flip-out clones (I,J). Phm levels in flip-out clones co-expressing DHR3-RNAi with ftz-f1-RNAi (K,L) or E75-RNAi (M,N). Phm and EcR expression in EcR-RNAi flip-out clone (O-Q). Phm levels in flip-out clones co-expressing EcR-RNAi with UAS-DHR3 (R,S), E75-RNAi (T,U) or ftz-f1-RNAi (V,W). Scale bars: 20 µm. For clonal analysis the stages of heat shock and dissection are indicated as in Fig. 3.
Ftz-f1 and EcR exert a forward effect to modulate DHR3 activity

To understand how EcR and βFtz-f1 restrain DHR3 expression in late L3 larvae, we first determined the window of time during which this regulatory process can be induced. Given that RNAi silencing is fully efficient 20 h after clonal recombination (supplementary material Fig. S10A-D), flip-out clones were induced at the L2/L3 transition and analysed 48 h later, before the L3/pupal transition. In this setting, DHR3 expression remained unaffected in either EcR-RNAi or ftz-f1-RNAi flip-out clones (Fig. 6A-D), whereas we previously showed an increase in DHR3 levels in early-induced clones (Fig. 3D-F and Fig. 4B,C). These findings indicate that both EcR and βFtz-f1 act early in development, to restrain DHR3 expression at the late L3 stage. Considering that EcR is required to maintain βftz-f1 expression in late L2 larvae (Fig. 3G-I), EcR might act through βftz-f1 to restrain DHR3 expression in late L3 larvae. Nevertheless, we wonder whether βftz-f1 could regulate EcR expression through a potential feedback loop. To address this issue, EcR levels were monitored in ftz-f1-RNAi flip-out clones. No effect were observed at mid-L2 stage (data not shown), while at late L2 stage EcR levels increased in the ftz-f1-RNAi clonal cells (Fig. 6E,F), indicating that depending on the development stage, βftz-f1 may impede EcR expression. Consistently, EcR levels dropped dramatically in UAS-βftz-f1 flip-out clones analysed at L2 or L3 stages (Fig. 6G-J). By contrast, most of the ftz-f1-RNAi clonal cells (70%) exhibited a mild decrease in EcR levels in early L3 larvae (Fig. 6K,L and data not shown); this phenotype was fully penetrant at the end of the L3 stage (Fig. 6M,N), indicating that βFtz-f1 is required to maintain EcR expression at this stage. Taken together, our findings unravel Fig. 6. Conditional misexpression of the NRs in the ring gland during development. (A-V) Flip-out clones labelled by GFP (A,C,E,G,J,K,M,O,Q,S,U, arrowheads in B,D,F,H,J,L,N,P,R,T,V) were stained for DHR3 (B,D), EcR (F,H,J,L,N,V) or Phm (P,R,T,V) protein. DHR3 protein levels in EcR-RNAi (A,B) or ftz-f1-RNAi (C,D) flip-out clones. (E-N) EcR protein levels in flip-out clones expressing ftz-f1-RNAi (E,F,K-N) or UAS-βftz-f1 (G-J) analysed at L2 or L3 stages. Phm protein levels in flip-out clones expressing E75-RNAi (O,P), ftz-f1-RNAi (Q,R), UAS-DHR3 (S,T). Phm and EcR protein levels in flip-out clones co-expressing EcR-RNAi and UAS-DHR3 (U,V). For clonal analysis the stages of heat shock and dissection are indicated as in Fig. 3. (W) Quantification of the phenotypes produced by various transgenes induced in the PG following the temperature shift to 29°C at the L2/L3 transition. The induced transgenes are indicated from left to right: control; EcR-RNAi, E75-RNAi, ftz-f1-RNAi, DHR3-RNAi, UAS-DHR3. Phenotype quantification is represented as in Fig. 2B (n: number of animals analysed).
an unexpected epistatic hierarchy between βFtz-f1 and EcR. βFtz-f1 exerts a negative input on EcR expression past the L2 ecdyson peak and a positive input in L3 larvae. Potentially, as the repression of EcR expression occurs when βFtz-f1 is highly expressed (Fig. 1A), it is conceivable that high βFtz-f1 levels repress EcR expression. In summary, although it is likely that EcR and βFtz-f1 act early through a common mechanism to restrain DHR3 expression at late L3 stage, it is not possible to decipher the hierarchy of this regulation.

EcR plays a dual role in the PG, as it is necessary for the DHR3-mediated repression of Phm and for limiting DHR3 expression in late L3 larvae. Our findings reveal an apparent paradox: we observed a decrease in both Phm and EcR levels in ftz-f1-RNAi clone out cells of late L3 larvae (Fig. 5A,B and Fig. 6M,N), while we provide evidence that EcR is required for the repression of Phm (Fig. 5S,T). We therefore hypothesized that, when it comes to restricting DHR3 expression, EcR may be required early in development to potentiate Phm downregulation at the late L3 stage. To address this issue, we induced flip-out clones at L2/L3 transition and monitored Phm expression 48 h later. We observed a severe drop in Phm levels in E75-RNAi but not in ftz-f1-RNAi clone out cells (Fig. 6O-R). Together with previous studies (Reinking et al., 2005; White et al., 1997), these findings suggest that E75 counteracts DHR3 activity, whereas βFtz-f1 acts early to restrict DHR3 expression and subsequently to sustain Phm expression. In addition, we observed that UAS-DHR3 downregulated Phm expression in PG cells of late L3 larvae, whether or not EcR-RNAi was co-expressed (Fig. 6S-V; supplementary material Fig. S9Q-S), suggesting that EcR is not required during the second half of the L3 stage for the DHR3-mediated repression of Phm.

Finally, we wondered whether the developmental arrests induced by NR misregulation depend on an early effect. To this end, we used the phm-gal4; tub-gal80Δ driver to delay the knockdown in the PG. Animals were reared at 18°C, then EcR-RNAi, E75-RNAi, ftz-f1-RNAi, DHR3-RNAi and UAS-DHR3 were induced following a temperature shift to 29°C at L2/L3 transition. In this setting, all the transgenes were highly effective 15 h past the temperature shift, although small amounts of DHR3 and βFtz-f1 can still be detected (supplementary material Fig. S1O-E-W). Developmental analysis revealed that roughly 40% of the larvae expressing either EcR-RNAi or ftz-f1-RNAi enter metamorphosis (Fig. 6W), further supporting that βFtz-f1 and EcR act, at least in part, through an early input to control the next ecdyson peak. By contrast, all of the larvae expressing either E75-RNAi or UAS-DHR3 were blocked at the late L3 stage (Fig. 6W), suggesting that these two NRs acts during the L3 stage to control the concurrent molting transition. Nevertheless, DHR3 also appears to act through an early input, as the quasi-totality of DHR3-RNAi expressing larvae underwent pupariation (Fig. 6W).

**DHR3 represses steroid synthesis**

To ascertain that DHR3 may act as a repressor of 20E synthesis, we measured ecdysonoid titres in larvae overexpressing DHR3 in their PG using the phm-gal4. Compared with control larvae, overexpression of DHR3 totally abolished steroid production at mid L2 stage (Fig. 7A). To analyse ecdysonoid titres in L3 larvae the tub-gal80ts;phm-gal4 driver was used to trigger transgene expression 24 h past the L2/L3 transition by switching animals from 18°C to 29°C. In this way, DHR3 overexpression induced a total arrest at late L3 stage (data not shown) and a severe drop in steroid titres at the timing of prepupal transition (Fig. 7B). Importantly, the steroid levels in these arrested larvae did not increase 24 h and 48 h later (Fig. 7B), indicating that DHR3 overexpression abolishes the ecdysonoid peak responsible for the prepupal transition. By contrast, triggering DHR3-RNAi 24 h past the L2/L3 transition did not impede metamorphosis onset (data not shown). Consistently, ecdysonoid titres in DHR3-RNAi animals remained unaffected at the prepupal transition, but significantly increased 3 h later (Fig. 7C). Taken together, these findings demonstrate that DHR3 is a bona fide repressor of steroid production.

**DISCUSSION**

**Unconventional 20E signalling regulates steroidogenic activity of the PG**

During the past few years, the *Drosophila* steroidogenic tissue has been extensively used to investigate how extrinsic and intrinsic parameters integrate to coordinate growth with developmental progression. Here, we show that EcR, E75, DHR3 and βFtz-f1, which mediate ecdysone signalling (King-Jones and Thummel, 2005), are expressed in the steroidogenic cells and are required for ecdysone synthesis during *Drosophila* larval development. These findings are consistent with previous studies showing that E75A mutation and βFtz-f1 disruption in the PG induce developmental arrest as a consequence of steroid deficiency (Bialecki et al., 2002;
Talamillo et al., 2013), and that DHR3 downregulation leads to late L3-arrested larvae (Caceres et al., 2011). In addition to E75, DHR3 and βFtz-f1, our study reveals that disrupting EcR in the PG also leads to developmental arrest that can be rescued by ecdysone feeding. To investigate the role of this NR cascade in the steroidogenic tissue, we generated somatic clones (Xu and Rubin, 1993) so that the steroid titres are maintained in the whole animal, but some cells are defective for a given NR. In this way, we observed that flip-out clones expressing a dominant negative form of EcR do not affect Phm expression, whereas a recent study reported that expression of the same EcR variant in the whole PG represses steroidogenic enzyme expression (Moeller et al., 2013). This discrepancy suggests that affecting 20E regulators in the entire PG induces a systemic response and does not allow studying cell-autonomous regulations.

Gene expression analysis following 20E stimulation supports a model in which the ternary complex EcR/USP/20E activates the transcription of E75 and DHR3, which together control the delayed expression of βFtz-f1 (King-Jones and Thummel, 2005). Our findings confirm that the dynamics of βFtz-f1 expression relies on DHR3 stimulation (Kageyama et al., 1997; Lam et al., 1999; White et al., 1997), although we observed that βFtz-f1 can be induced later in DHR3 mutant cells. Moreover, EcR is dispensable for DHR3 expression in the steroidogenic tissue of L3 larvae raises the question of whether an EcR-independent 20E signalling (Srivastava et al., 2005) is active in PG cells. Unexpectedly, disrupting E75 or βFtz-f1 provokes developmental arrest at the end of the L2 stage, whereas disrupting EcR or DHR3 provokes developmental arrest at the end of the L3 stage. Moreover, downregulating DHR3 can rescue L2 arrest due to disruption of either E75 or βFtz-f1, which further supports the idea that an unconventional NR cascade regulates steroidogenesis. Alternatively, as these NRs respond to 20E cyclic production and can act through a delayed effect in PG cells (see below), it is possible that disrupting the 20E response at a given peak may affect the 20E response at the next peak, thus leading to an apparent unconventional response. Nonetheless, our findings place DHR3 rather than βFtz-f1 (Ou and King-Jones, 2013; Parvy et al., 2005; Rewitz et al., 2013; Yamanaka et al., 2013) as the downstream effector of the NR cascade in the control of steroid production (Fig. 7D).

**DHR3 mediates a feedback control on steroid biosynthesis**

Our study reveals that DHR3 represses steroidogenesis both at the L2 and L3 stages. At the late L3 stage, DHR3 downregulates expression of the steroidogenic enzymes Phm, Dib and Sad. Several studies describe DHR3 as a transcriptional activator whose activity is inhibited by E75 (Caceres et al., 2011; Palanker et al., 2006; Reinking et al., 2005; White et al., 1997), suggesting that DHR3 may act indirectly to repress steroidogenesis. We previously reported that βFtz-f1 is necessary for Phm and Dib expression (Parvy et al., 2005). Here, we show that βFtz-f1 does not directly activate the transcription of these enzymes, but acts by restraining DHR3 expression. Furthermore, we provide evidence that E75 also acts to restrict DHR3-mediated repression of Phm. Hence, DHR3 emerges as a major repressor of steroidogenesis. Nonetheless, discriminating between direct transcriptional suppression and indirect repression through the activation of an unknown intermediate suppressor will require an in-depth analysis of the direct DHR3 DNA targets.

The increased expression of Phm and Dib in DHR3 mutant cells together with the increased ecdysoid levels in DHR3 deficient animals past the L3/prepupal transition indicates that DHR3 acts in a feedback loop to sharpen the peak of ecdysone (Fig. 7D). Consistent with this, previous works revealed that progression through metamorphosis requires 20E inactivation, an enzymatic reaction catalysed by Cyp18A1 (Guitard et al., 2011; Rewitz et al., 2010). These observations underscore the notion that the transient increase of ecdysone titres is as critical as the inactivation/clearance of the hormone. However, the fact that the development of Cyp18a1 mutants is not arrested earlier than the prepupal stage (Guitard et al., 2011; Rewitz et al., 2010) and that DHR3 does not repress the steroidogenic enzymes at the late L2 stage suggests that the drop in 20E titre is more critical at the onset of metamorphosis than it is for the L2/L3 transition. This discrepancy may be a consequence of the different 20E titres at the L2 versus late L3 stage, or alternatively because a larva excretes organic compounds, whereas a prepupa is a closed system (Lafont et al., 2012).

Our findings suggest that EcR acts through a mechanism that occurs early in development to potentiate the DHR3-dependent Phm downregulation at late L3 stage. In contrast to a previous study (White et al., 1997), it is unlikely that EcR and DHR3 physically interact in this downregulation, as the DHR3-mediated repression of Phm is observed even when EcR is knockdown from early L3 stage. Interestingly, EcR has been shown to work as a modifying cofactor of the chromatin structure (Sedkov et al., 2003). Moreover, in mammalian steroidogenic tissues, specific histone modifications are associated with the rapid increase in expression of the steroidogenic acute regulatory protein (StAR), the rate-limiting factor of steroidogenesis (Hiroi et al., 2004a,b). Therefore, it is conceivable that once past the L2 ecdysone peak, EcR might induce chromatin modifications, allowing DHR3 to mediate Phm repression at the L3/prepupal transition. At this stage, this repression will stop ecdysone biosynthesis so that metamorphosis can proceed.

**A forward control restrains the DHR3-mediated repression of steroidogenesis**

Concerning the L3/prepupal transition, our study indicates that βFtz-f1 and EcR act early in development to regulate DHR3 expression. By contrast, E75-RNAi and DHR3 overexpression provokes a developmental arrest at the end of the stage concurrent to their induction. These findings revealed that early and concurrent regulatory processes control steroidogenesis. Interestingly, we provide evidence that DHR3 act through both processes, as its downregulation provokes an L3 developmental arrest when knockdown is induced early in development but not past the L2/L3 transition. In addition, E75A overexpression provokes a phenotype similar to the one induced by DHR3 knockdown. Therefore, as shown in previous studies (Palanker et al., 2006; Reinking et al., 2005; White et al., 1997), E75A might directly counteract DHR3 activity to regulate steroidogenesis. Furthermore, E75 has been proposed to sustain and amplify ecdysone production through a feed-forward effect (Bialecki et al., 2002) suggesting that the developmental arrest observed in E75A mutants is due to the defect in ecdysone auto-amplification. In light of our study, the developmental arrest of E75A mutants is likely to be a direct consequence of premature DHR3-repressing activity, whereas our observation of early induced events unravels a step-forward process. This step-forward effect roughly originates soon after the L2 20E peak to moderate later DHR3 expression. Earlier studies have reported that in the salivary glands, the response to 20E requires a gap to acquire competence for re-induction (Richards, 1976a,b) and that βFtz-f1 is a competent factor in this process (Broadus et al., 1999; Woodard et al., 1994). Competence is described as the acquired ability of one group of cells to respond to a developmental...
signal, while other cells do not respond to this signal (Waddington, 1940). In the case of 20E responsiveness, the gap for acquisition of competence is associated with low ecdysone titres and high βFtz-f1 levels. Our findings support the notion that βFtz-f1 also acts as a competent factor for ecdysone biogenesis through a step-forward moderation of DHR3 expression. In addition, as both EcR and DHR3 knockdown also act through an early induced event and affect βFtz-f1 expression, it is conceivable that they participate in the molecular mechanisms that link βFtz-f1 and EcR to DHR3 must still be elucidated, our study reveals that the response following the L2 ecdysone peak is necessary to confer competence for ecdysone biogenesis at the late L3 stage by delaying the DHR3-mediated repression of steroidogenic enzymes.

In summary, our study unravels an autoregulatory mechanism in cyclic ecdysone production. This autoregulation is likely to be coordinated with the processes that adjust ecdysone biogenesis at the L3 stage in response to environmental cues. These include nutrition, insulin signalling and the circadian rhythm that integrates through the prothoracicotropic hormone (PTTH) (Caldwell et al., 2005; Colombani et al., 2005; Layalle et al., 2008; McBrayer et al., 2007; Mirth, 2005). Interestingly, a downstream effector of PTTH is the NR Ftz-f1 (Parvy et al., 2005) using the L2 antiserum. Each determination was made on two UAS-DHR3 in Fig. 7A) or at least five replicates and the results were expressed as mean values+s.e.m. For each replicate, 20 (Fig. 7A) or 10 (Fig. 7B,C) animals were used.

ECYSDEROID FEEDING AND MEASUREMENT
Ecdyscoid feeding rescue was performed using 1.5 ml of warm-melted medium mixed with either 50 μl or 150 μl of a 20E stock solution (10 mg/ml 20E in 95% ethanol) for L2 to L3 rescue or L3 to pupal rescue, respectively. Controls contained either 50 μl or 150 μl of a 95% ethanol solution. For 20E rescue mid L2 or late L3 larvae were transferred on appropriate media, and the larvae that underwent molting transition were counted the following day.

Ecdyscoid extracts were obtained and purified as previously described (Parvy et al., 2005) using the two independent groups of 20 larvae for each time point.

COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
J.-P.P. and J.M. conceived the study, designed and analysed experiments, wrote the manuscript. All authors performed experiments.

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