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ORIGINAL ARTICLE

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Parallel alterations in the timing of ovarian Ecdysone Receptor and Ultraspiracle expression characterize the independent evolution of larval reproduction in two species of gall midges (Diptera: Cecidomyiidae)

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Abstract Although most insects reproduce in the adult stage, facultative larval or pupal reproduction (paedogenesis) has evolved at least six times indepently in insects, twice in gall midges of the family Cecidomyiidae (Diptera). Paedogenesis in gall midges involves the precocious growth and differentiation of the ovary in an otherwise larval form. We have previously shown that the timing of expression of the Ecdysone Receptor (EcR) and Ultraspiracle (USP), the two proteins that constitute the functional receptor for the steroid hormone 20-hydroxyecdysone, regulates the timing and progression of ovarian differentiation in *Drosophila melanogaster* (Diptera: Drosophilidae). Here we test the hypothesis that precocious activation of EcR and USP in the ovaries of paedogenetic gall midges allows for precocious ovarian differentiation. Using monoclonal antibodies directed against insect EcR and USP proteins, we first show that when these gall midges are reared under conditions that promote typical, metamorphic development, up-regulation of EcR and USP occurs in the final larval stage. By contrast, in the paedogenetic life cycle, EcR and USP are up-regulated early in the first larval stage. A similar pattern is seen for two independently-evolved paedogenetic gall midges, *Heteropeza pygmaea* and *Mycophila speyeri*. We discuss our results in the context of developmental constraints on the evolution of paedogenesis in dipteran insects.

Key words Paedogenesis · Ovary · Heterochrony · Homoplasy · Development and evolution

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Introduction

There are many instances in metazoan evolution of radical alterations in life histories. Among the most striking and commonly encountered patterns of this kind are the multiple examples of the evolutionary loss of larval feeding (lecithotrophy) within groups generally characterized by a feeding larval stage (reviewed in Strathmann 1985). In the echinoids (sea urchins and their relatives) alone, lecithotrophy, via the loss of the feeding pluteus larval stage, has probably evolved at least 21 times independently (G. Wray, personal communication). The evolution of lecithotrophy is generally characterized by increased egg size (with the additional maternally-deposited material substituting for larval feeding) and a shorter period from embryogenesis to the juvenile stage (reviewed in Wray 1995). In this way, lecithotrophs have undergone a heterochronic shift (alteration in developmental timing; reviewed in Gould 1977) in the onset of juvenile development (Raff 1987). Among echinoids, such life history shifts may have evolved in as few as 4–8 million years (McMillan et al. 1992; Wray 1992).

The mechanisms underlying such evolutionary transformations are beginning to be elucidated. Ascidians (which do not feed, but generally disperse as larvae) have probably lost the swimming tadpole form on at least four independent occasions (Hadfield et al. 1995). One well-studied species pair is *Molgula oculata* and *M. occulta*, in which the former has a typical tadpole stage, while the latter lacks many tadpole features, such as the tail and sense organs (reviewed in Jeffery and Swalla 1992). Recently, Swalla and Jeffery (1996) have shown that the zinc finger gene *Manx* is required for the differentiation of these tadpole features in hybrid embryos, and is not expressed during embryogenesis in *M. occulta*. Amphibians also typically develop through a tadpole stage, and direct development, characterized, for example, by the precocious differentiation of limb primordia in the embryo and subsequent skipping of the tadpole stage, has evolved multiple times (reviewed in Hanken et al. 1997). Amphibian metamorphosis is regulated by thyroid hormones

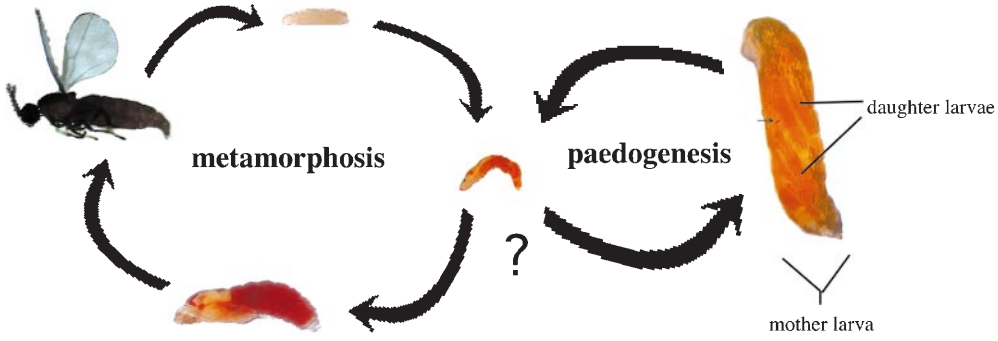

Fig. 1 Life cycle of *Mycophila speyeri*. All pictures are at the same scale. The large orange larva *on the right* of the figure is a mother larva brooding her daughter larvae (distinguishable by the dark eye-spots; *arrowhead*). The ontogenetic mechanism causing the switch between the two life cycles is not well understood (*question mark*). The independently evolved paedogenetic life cycle of *Heteropeza pygmaea* is remarkably similar

(reviewed in Tata 1996). Jennings and Hanken (1998) have shown that the thyroid gland becomes active in late embryogenesis in the direct-developing frog *Eleutherodactylus coqui*, while the thyroid glands of metamorphic amphibians do not begin to differentiate until tadpole stages. Furthermore, thyroid hormone receptors appear in embryonic limb buds of *E. coqui*, while this expression is not detected at similar stages in the limb buds of *Rana pipiens*, a metamorphosing frog (Hanken et al. 1997). Alterations in the thyroid hormone axis also typify the loss of metamorphosis in neotenic salamanders (Frieden 1981; Yaoita and Brown 1990). In each of these instances, an apparently simple heterochronic change may underlie radical morphological and life history transformations, as was hypothesized by Gould (1977).

In insects, direct development is the apparent ancestral condition. The more derived, holometabolous insects (flies, bees, butterflies, etc.) are a monophyletic assemblage (Kristensen 1991) in which complete metamorphosis is a shared feature. There have been no instances found of an evolutionary loss of metamorphosis within the Holometabola. However, several gall midges (Diptera of the family Cecidomyiidae) and one beetle (*Micromalthus debilis*, family Micromalthidae) have a facultative, larvally-reproductive life cycle known as paedogenesis (Wagner 1862; Felt 1911; Barber 1913; Scott 1938; Wyatt 1961, 1963, 1964, 1967). In typical holometabolous insects, the ovaries differentiate during the end of larval development and metamorphosis (Büning 1994). By contrast, in the paedogenetic life cycle, the ovaries differentiate and grow precociously in the early larval stage. The eggs activate parthenogenetically, and the embryos are brooded inside the mother larva's hemocoel. Ultimately the larvae hatch, consume the histolyzing tissues of the mother, and emerge from the mother's empty cuticle. In paedogenetic gall midges, if fungal food resources remain plentiful, the larvae will repeat the paedogenetic life cycle. When conditions worsen, the larvae will develop through metamorphosis, and fly away to find another good fungal patch (Fig. 1; reviewed in Went 1979). Because reproduction occurs precociously, the paedogenetic life cycle is very rapid, in some species as short as four days (Ulrich et al. 1972).

What is the mechanism by which the ovaries alone can differentiate inside an otherwise larval form? Metamorphic events in insects are known to be regulated by the molting hormone 20-hydroxyecdysone (20 E) and its active metabolites (Riddiford 1985), presumably via the action of the Ecdysone Receptor (EcR) and its heterodimeric partner Ultraspiracle (USP) (Yao et al. 1992, 1993). Studies by Went (1978) on the in vitro development of the ovaries of the paedogenetic gall midge *Heteropeza pygmaea* demonstrated that 20 E accelerated the formation of follicles 2-fold, while juvenile hormone III (JH III) and farnesol (a JH precursor) inhibited follicle formation. Treiblmayr et al. (1981) reported a similar result with topical application of 20 E in vivo. Interestingly, the stage during normal paedogenetic development at which the somatic cells of the ovaries begin to differentiate is just after the time of the highest whole-animal titers of 20 E (Went et al. 1984). In *Drosophila melanogaster*, 20 E has also been implicated in regulating ovarian differentiation (Audit-Lamour and Busson 1981), which begins as the larva prepares for metamorphosis (Kerkis 1931, 1933), as is the case in most holometabolous insects (Büning 1994). In *D. melanogaster*, EcR and USP colocalize in seven of the eight mesodermally-derived somatic ovarian cell types as they differentiate, with high levels of USP expression tending to correlate with low relative levels of EcR, and vice versa (Hodin and Riddiford 1998). The one exception to colocalization is the terminal filament cells, the first cell type to differentiate (King et al. 1968; Godt and Laski 1995; Hodin and Riddiford 1998), in which USP is expressed in the absence of EcR. The germ cells express neither EcR nor USP at any time during ovarian differentiation (Hodin and Riddiford 1998). Flies carrying mutations in *EcR*, *usp*, or both are defective in the timing and progression of ovarian differentiation, suggesting that this process is at least in part regulated by the timing of expression of the *EcR* and *usp* genes (Hodin and Riddiford 1998).

Here we test the hypothesis that a change in the timing of *EcR* and *usp* expression allows for precocious ovarian differentiation in paedogenetic gall midges, using two species of gall midges in which paedogenesis has evolved independently. We first show that when these flies develop through metamorphosis (when the larvae are reared on poor-quality food), EcR and USP expression correlates with ovarian differentiation at metamorphosis. We further demonstrate that precocious up-regulation of EcR and USP in larval ovarian cells correlates with the shift into the paedogenetic life cycle

in both gall midge species examined. We discuss these results as an example of an apparent developmental constraint on the evolution of larval reproduction in insects.

Materials and methods

Animal stocks

Laboratory strains of the cecids *Mycophila speyeri* Barnes and *Heteropeza pygmaea* Winnertz derived from stocks collected in 1984 by I.J. Wyatt at a mushroom farm in Littlehampton, West Sussex, England. Larvae were reared on pure mycelial cultures of the mold *Peniophora albula* grown on a malt agar medium [0.5% Malt Extract Broth (Difco) and 2% Bacto Agar (Difco) in distilled water] in 60×15 mm Petri plates. "Fresh food" is mycelium that was allowed to grow for 3 days at 25 °C, with the surface of the agar not completely colonized by the mold (Fig. 2 A). Fresh food was stored for less than 1 month at 4 °C. "Old food" was left at 25 °C or room temperature (RT) for at least 7 days, until the mycelium took on a dry, clumpy appearance (Fig. 2B), and had a musty aroma. Cecid stocks were maintained at RT, or kept in long term culture at 12 °C (at this temperature they enter a relatively dormant state, and can be stored for 2 months or more). Experiments were performed at 25 °C.

Staging and dissections

The "red eye" stage is the time when the eyes of the daughter larvae are first clearly visible using a dissecting microscope. We define "birth" as the time when the first daughter larva emerges from the mother. Final instar larvae are characterized by the presence of a "sternal spatula," a darkly pigmented, cuticular structure found on the anterior-ventral surface of the animal. Larvae which have this structure are destined for metamorphosis (Wyatt 1963, 1967).

Larvae and pupae were dissected in a depression slide in phosphate-buffered saline (PBS: 130 mM NaCl, 10 mM $NaPO_4$, pH 7.0) using jewelers forceps and tungsten needles. The larvae were torn in half and both the anterior and posterior halves were squeezed so the internal organs protruded from the cut site. The dissected larvae were then placed into staining chambers, consisting of an inverted screw-top microcentrifuge tube in which the bottom had been cut in half, the cap had a hole cut in it, and a piece of 32 or 53 µm Nitex mesh was screwed between the cap and the top half of the tube. These chambers fit nicely into 4-well microtiter plates (Nunc) for staining reactions. Dissected tissues were maintained in PBS on ice until fixation. Brains and ovaries from wandering *D. melanogaster* larvae were used as positive controls, and were processed together with the ovaries.

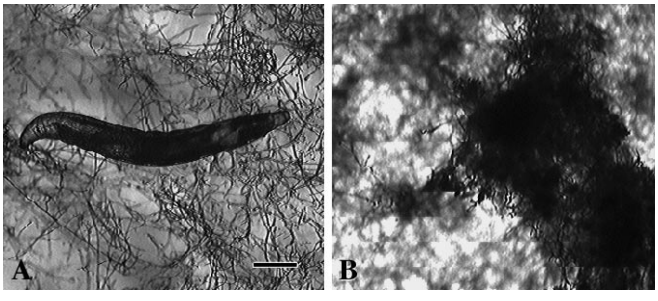

Fig. 2A, B Different types of food used in this study. **A** Fresh food. Notice the stringy appearance of the mycelium being consumed by this *M. speyeri* larva. The *scale bar* is 0.1 mm. **B** Old food. Notice the typical, clumpy appearance of the mycelium. Same magnification as in **A**

Immunocytochemistry, confocal microscopy and image processing

Larval and pupal tissues were fixed as previously described (Hodin and Riddiford 1998), except that the fixative included 1% Triton X-100. Tissues to be treated with antibodies were first blocked for at least 30 min at room temperature (RT) in 5% normal goat serum in PBT [PBS plus 1% Triton X-100] with 0.01% sodium azide, and then incubated in either *Drosophila* USP monoclonal antibody (1:200 AB11; Khoury-Christianson et al. 1992) or *Manduca sexta* EcR monoclonal antibody (1:100 15C3; Jindra et al. 1996) diluted in PBT with 0.01% sodium azide for either 3 h at RT or overnight at 4 °C. Next, tissues were washed nine times for 10 min in PBT, and incubated in 1:500–1:1000 donkey anti-mouse CY5 or FITC (Jackson Immunoresearch) secondary antibody in PBT with 0.01% sodium azide in the dark for either 3 hr at RT, or overnight at 4 °C. Samples were then double-labeled with either Bodipy-phalloidin 558/568 (1 unit/200 µl PBT; Molecular Probes) for 45 min at RT to visualize cortical actin (cell borders and ring canals), or 0.4 µg/ml propidium iodide in PBT for 5 min at RT to visualize nuclei, and then washed three times for 20 min in PBT. Tissues were mounted in 90% glycerol/1X PBS/0.01% sodium azide for confocal microscopy. Negative controls were processed as above, omitting the primary antibody step.

Confocal images were obtained using a BioRad MRC-600 Confocal laser scanner, an Optiphot Nikon microscope, Kalman filtering and COMOS software (BioRad). Images were processed using NIH Image (public domain) and Adobe Photoshop (Adobe Systems). Light micrographs were captured using a Sony CCD video camera mounted on a Nikon Optiphot light microscope, with or without DIC optics.

Head capsule measurements

Light microscopic images of the heads of actively feeding larvae were captured as described above, and head capsule width of the anterior-most head segment behind the mouth-parts was measured using Adobe Photoshop (Adobe Systems). T statistics and standard error calculations were performed using the Systat 5.2.1 software package.

Results

Developmental timing

When larvae of *H. pygmaea* or *M. speyeri* are reared on fresh food (see Materials and methods; Fig. 2A), all the animals undergo larval reproduction (paedogenesis). One complete paedogenetic life cycle (from the time of birth from the mother larva to the time at which the offspring themselves give birth) is 5 days in our *H. pygmaea* strain and 5.5 days in our *M. speyeri* strain [studies on different strains of *M. speyeri* placed the life-cycle length at 4.5 days (Schüpbach and Camenzind 1983) and 6 days (Wyatt 1964)]. Table 1 gives a complete timeline of paedogenetic development in the two species, and our results are largely consistent with previous reports (Went 1971; Schüpbach and Camenzind 1983). Briefly, in both species, morphological differentiation of the ovaries is apparent by 6–12 h after hatching, at which point follicle cells begin to surround the nascent follicles (Fig. 3, bottom). By 24 h, the follicles have grown approximately 5-fold, and are being released into the hemocoel. Embryogenesis ensues within the following 12–24 h.

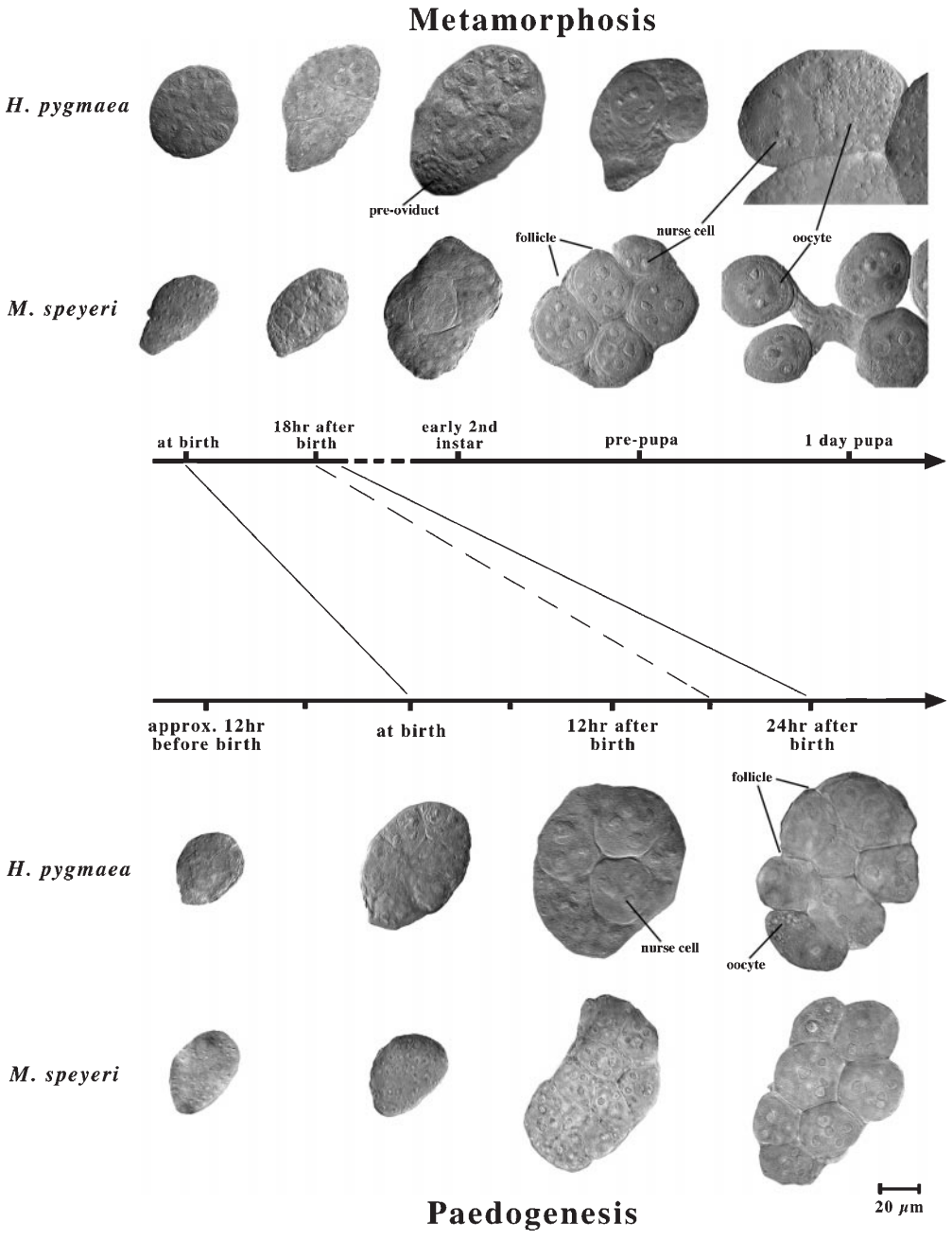

Fig. 3 Ovarian differentiation during metamorphic and paedogenetic development in both gall midge species. The time course of metamorphic ovarian development in the two species is similar (*upper panels*). During paedogenetic development (*lower panels*), ovarian differentiation and follicle formation occur in both species during the first day after birth. Note that by 24 h in both species, ovarian development is at a state as advanced as seen in the pre-pupal stage during metamorphic development (compare *upper and lower panels*), which occurs more than 5 days after birth. All ovaries are to scale

When larvae of *M. speyeri* are reared on old food (see Materials and methods; Fig. 2B), a variable proportion (usually about half; $n>100$ larvae; data not shown) of the larvae undergo metamorphosis (though those that eventually reproduce paedogenetically only do so after a delay of a few days). The time from birth to metamorphosis varies greatly among experiments. We presume that the variability in the quality of "old food" leads to the variability in the timing of the metamorphic response, as well as in the proportion of larvae responding. Table 2 gives a complete timeline of metamorphosis from the second larval instar through adult eclosion in *M. speyeri*. The growth and differentiation of the ovary during the metamorphic life cycle is shown at the top of Fig. 3. Follicles are first distinguishable in the early to mid second (final) instar.

When larvae of *H. pygmaea* are reared on old food, the metamorphic response is not nearly so robust as in *M. speyeri*. Typically, paedogenetic reproduction ensues but is always delayed by at least several days, and fewer embryos are brooded (see also Went 1975, 1979). Only very rarely (approximately 1% of the time; $n>300$) will the larvae enter metamorphosis. In this study we will refer to *H. pygmaea* "metamorphs" as those larvae reared under the conditions which produce this developmental delay. We have found no reliable way to predict when metamorphosis will occur before the larvae reach the final instar (see also Wyatt 1961; Went 1982), and express metamorphic-

Table 1 Timing of paedogenetic events in two gall midge species reared at 25 °C on fresh food

Age	*H. pygmaea*	*M. speyeri*
Approx. 12 h pre-birth	Mother's fat body fragmenting; mother inactive; red eyes of daughter larvae clearly visible; pre-hatching; ovary undifferentiated	Mother's fat body still largely intact; mother active; red eyes of daughter larvae clearly visible; pre-hatching; ovary undifferentiated
Approx. 6 h pre-birth	Daughter larvae hatched, active inside mother; fat body of mother fragmented	Mother larva still active; fat body in two stripes along lateral edges; daughter larvae hatched and active inside mother
2–3 h pre-birth	Cuticle of mother darkens (brownish tint)	Mother larva mostly inactive, fat body fragmented
At birth	Ovaries of daughter larvae unchanged in gross morphology from 12-h pre-birth	Ovaries of daughter larvae unchanged in gross morphology from 12-h pre-birth
6 h after birth	Follicle cells beginning to surround and separate follicles	No evidence yet of follicle separation
12 h after birth	Follicles more prominent	Follicle cells beginning to surround and separate follicles
18 h after birth	Follicles increased in size approximately 2-fold from birth	Follicles increased in size approximately 2-fold from birth
1 day after birth	Follicles increased in size approx. 5-fold from birth; some follicles already released from the ovary	Follicles increased in size approx. 5-fold from birth; follicles being released from the ovary
1.5 days after birth	Embryogenesis begins	Follicles growing
2 days after birth	Embryonic germ band visible	Embryogenesis begins
3 days after birth	Segmentation of embryos visible	Mother molts to second larval stage
4 days after birth	Mother mostly inactive, fat body intact	Segmentation of embryos visible
4.5 days after birth	Red-eye stage of next generation (see above)	Mother still active, fat body intact
5 days after birth	Birth of next generation	Red-eye stage of next generation (see above)
5.5 days after birth		Birth of next generation

Table 2 Timing of metamorphic events in *M. speyeri* at 25 °C on old food. The timing of events in the second instar should be taken as approximate, as this can vary substantially with food quality. Data for pupae are based on 10 animals. "P-2.5+" is 2.5 or more days before pupation, etc.

Age (days)	Description
P-2.5+	Pointed thin heads, sternal spatulae not entirely pigmented (early second instar)
P-2	Pointed thick heads, sternal spatulae entirely pigmented (mid second instar)
P-1.5	Thick rounded head, crawlers (late second instar)
P-0.5	Thick rounded head, cannot crawl but twirl (prepupa)
P+0	Post ecdysis, orange body
P+1	Body color pinkish
P+2	Body color more pink, fat body in head beginning to fragment, faint eye pigment
P+3	Reddish body color, eye pigment brown, tip of abdomen white
P+4	Eye pigment black, brown wings, white abdomen
P+5	Eclosion

Table 3 Timing of metamorphic events in *H. pygmaea* reared at 25 °C on old food

Age (days) / stage	Description
early second	Pointed thin heads (sternal spatulae not entirely pigmented during the first day of the final larval stage)
Mid second	Pointed thick heads
Late second	Thick rounded head, crawlers
Pre-pupa	Thick rounded head, cannot crawl but twirl
P+0	No eye pigment, white body color (fat body)
P+0.5	Tan eye pigment
P+1	Brown eye pigment
P+1.5	Brick eye pigment, yellowish abdomen
P+2	Almost-black eye pigment, orange abdomen
P+2.5	Black eye pigment, prominent white spot near posterior of abdomen
P+3–3.5	Larger white spot, wings grey-brown, posterior tip of abdomen grey-brown
P+3.5–4	Head capsule dark grey
P+4–5	Eclosion

specific markers (see Materials and methods). Table 3 gives a timeline of metamorphosis from the final larval instar through adult eclosion in *H. pygmaea*. We have no data on the timing of events in the second larval instar, because these animals were rarely encountered, and when available, were used to examine metamorphic ovarian differentiation (Fig. 3, top). Ovarian differentiation progresses similarly to that of *M. speyeri*, with differentiating follicles in *H. pygmaea* clearly distinguishable by the mid second (final) instar (not shown), slightly later than in *M. speyeri*. Yet in the pupal stage, oogenesis proceeds more rapidly in *H. pygmaea* (Fig. 3, top).

To determine the number of larval instars in the paedogenetic life cycle, we measured the head capsule width in five larvae at 1 day intervals starting at birth. In *H. pygmaea*, the head capsule width did not change significantly during the first 4 days after birth [approximately 72±2 µm standard error (S.E.)], although the larvae grew substantially during this period. By 4.5 days after birth, the head capsule width increased to approximately 81±2 µm S.E. (P<0.05), consistent with previous reports that around this time, *H. pygmaea* forms a "hemipupa" by secreting a new cuticle while retaining the old cuticle (Wyatt 1967). In *M. speyeri*, the head capsule width did not change during the first 2 days after birth (approximately 76±3 µm S.E.). By day 4, the head capsule width increased to 92±5 µm S.E. (P<0.05), and did not change significantly until those larvae themselves gave birth. Therefore our observations are consistent with previous reports that *M. speyeri* reproduces paedogenetically as a second instar larva (Wyatt 1964; Ulrich et al. 1972).

Because we could not identify which larvae reared on old food were destined for metamorphosis, we could not similarly determine the number of larval instars in the metamorphic life cycles of these insects. Still, the head capsule widths of final larval instar metamorphs of both species (n=5; 102±7 µm S.E. in *H. pygmaea*; 93±1 µm S.E. in *M. speyeri*) were significantly larger than in first instar larvae (P<0.02 for both species), suggesting that pupation occurs after the second larval instar in both species. This finding for *M. speyeri* is consistent with a previous report (Wyatt 1964; although he reported that *M. speyeri* will occasionally insert a third larval stage in the metamorphic life cycle). However, Wyatt (1963) concluded that first instar larvae molt directly to the pupal stage in the metamorphic life cycle of *H. pygmaea*. We consider this odd, as the metamorphs clearly gain the cuticular sternal spatula during larval development, suggesting that there is at least one larval molt. For this reason, and from our data on head capsule widths, we will refer to the final larval stage (before pupation) as the second larval stage in both species, defined by the presence of a sternal spatula (see Tables 2 and 3).

USP and EcR localization during metamorphic development in *H. pygmaea*

When *H. pygmaea* larvae are reared on old food and undergo metamorphosis, the ovaries do not differentiate into follicles until the second (final) larval instar (Fig. 3). To determine if differentiation of the ovaries at this stage correlates with USP and EcR expression, we immunostained ovaries with USP- and EcR-specific monoclonal antibodies from birth through early pupal stages in *H. pygmaea* larvae reared on old food (Fig. 4).

The *Drosophila* USP monoclonal antibody detects an epitope in the region just upstream of the first zinc finger (Khoury-Christianson et al. 1992), a region that is conserved among all arthropod USPs and RXRs (which are orthologous genes) sequenced to date (Hayward et al. 1999). This antibody detects a nuclear antigen in both the brain (Fig. 6; not shown for *M. speyeri*) and ovary (Figs. 4, 5, 7) in both gall midge species. We tested a series of six *Drosophila (*Talbot et al. 1993) and two *Manduca* (Jindra et al. 1996) EcR monoclonal antibodies directed against the common regions of these proteins. Only one of these (*Manduca* 15C3) gave nuclear staining in both the brain (Fig. 6) and the ovary (Figs. 4, 5) of both gall midge species (not shown for *M. speyeri*). Nuclear localization was confirmed by double-labeling *H. pygmaea* and *M. speyeri* ovaries with propidium iodide, a nuclear stain (Figs. 4, 5, 7). All staining for both antibodies was nuclear, with the exception of occasional perinuclear staining with the EcR antibody in some experiments, particularly in pupal ovaries (not shown). This latter staining was presumably artifactual. The fact that the nuclear staining patterns with the USP and EcR antibodies were similar in both the brain (Fig. 6) and the ovary (Figs. 4, 5) suggests that the staining patterns that we report represent gall midge USP and EcR.

We detected low levels of both USP (Fig. 4A) and EcR (Fig. 4E) in a subset of the somatic cells of *H. pygmaea* ovaries at birth (the stage at which the daughter larvae emerge from the mother larva). We are not certain what the fates of these cells are, but we suspect that they are pre-follicle cells (Went 1982; see below). Since we could not obtain reliable double-labels with the two monoclonal antibodies, we could not confirm if the same cells that expressed USP also expressed EcR, though their locations in the ovary were similar. At 18 h after birth, the expression patterns had not changed markedly (Fig. 4B, F). Rarely, a few larvae would molt to a second (final) instar larva, and proceed towards metamorphosis (see Table 3 for staging criteria). Early second larval stage ovaries continued to show only low levels of USP and EcR expression (not shown, but similar to Fig. 4C, G). By the middle of this instar, we detected high levels of USP in the somatic cells. At this stage, the follicle cells and the pre-oviduct cells (Büning 1994) expressed very high levels of USP (Fig. 4C). At the pre-pupal stage, the differentiated follicle cells and fully formed anterior oviduct continued to express EcR and high levels of USP (Fig. 4D, H). We did not detect USP expression in the nurse cells until the pre-pupal stage (Fig. 4D). We could not determine whether EcR was similarly expressed in these cells due to high background (cytoplasmic and perinuclear; see below) staining of EcR in pre-pupal (Fig. 4H) and pupal (not shown) egg cham-

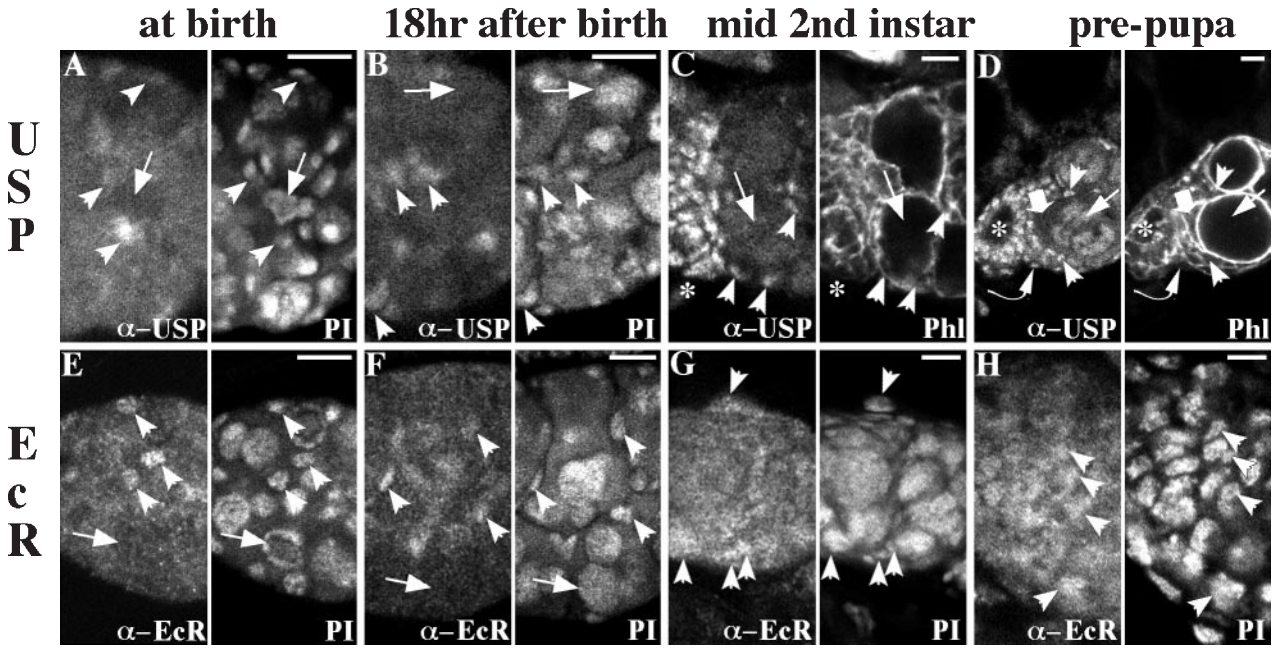

Fig. 4 USP (**A–D**) and EcR (**E–H**) expression in the ovary during metamorphic development in *H. pygmaea*. *PI* = propidium iodide, which stains nuclei. *Phl* = phalloidin, which stains cortical actin (cell borders). At birth, USP (**A**) and EcR (**E**) were detected in mesoderm-derived somatic cells (*arrowhead*), but not in the nurse cells (*arrow*). The brighter staining nuclei are presumably the follicle stem cells. **B**, **F** 18 h after birth. The patterns of USP (**B**) and EcR (**F**) expression were similar to that seen at birth (symbols as in **A**, **E**). **C**, **G** Mid second instar. High levels of USP (**C**) expression were seen in the presumptive oviduct (group of cells *at the far left, above the asterisk*) and follicle cells (*arrowheads* point to follicle cells surrounding a single follicle). Nurse cells had no detectable USP (*arrow*). EcR expression (**G**) was faint at this stage, but detectable in the follicle cells (*arrowheads*). **D**, **H** Pre-pupa. High expression of USP (**D**) was maintained in the fully-formed oviduct (*asterisk*) and follicle cells (*arrowheads*, as in **C**). USP was present in the nurse cells at this stage (*straight thin arrow*) but not in the oocyte (*straight wide arrow*). The sheath cells also expressed USP (*curved arrow*). EcR (**H**) was still detectable in the follicle cells (*arrowheads*). All *scale bars* are 10 μm

bers. EcR and USP patterns did not change appreciably as the follicles continued to grow in the pupal stage (not shown). No USP (Fig. 4D) or EcR (not shown) staining was ever seen in the oocyte nucleus.

In summary, we detect low relative levels of USP and EcR in the ovary during early larval stages in *H. pygmaea* metamorphs. Then, as the larvae begin to enter metamorphosis, EcR and USP are up-regulated in the ovary as it begins to undergo its dramatic metamorphic changes.

USP and EcR localization during paedogenetic development in *H. pygmaea*

When *H. pygmaea* larvae are reared on fresh food, ovarian differentiation is morphologically apparent by 6–12 h after the daughter larvae emerge from their mother larva (Fig. 3; Table 1). To determine if precocious ovarian differentiation in these paedogenetic larvae correlates with alterations in the patterns of USP and EcR expression, we immunostained ovaries with USP- and EcR-specific antibodies from 12 h before birth until 24 h after birth (at which time follicles are already being released from the ovary).

At 12 h before birth, we could not detect USP or EcR at levels above background in the undifferentiated ovaries, though these proteins were expressed in both the brain and epidermis at this stage (not shown). At birth, both USP (Fig. 5 A) and EcR (Fig. 5D) were expressed in a subset of the somatic cells of the ovary, in a pattern similar to that described above for *H. pygmaea* metamorphs at birth (Fig. 4A, E). Although we cannot directly compare levels of expression, it appeared that USP stained more strongly (and in more cells) in the paedogenetic ovaries at birth (compare Figs. 4 A and 5 A). By 6 h after birth, USP was expressed in follicle cells as they began to surround nascent follicles (not shown). By 12 h, both USP and EcR continued to be expressed more broadly in the somatic cells, as follicle cells had completely surrounded the nascent follicles (Fig. 5B, E; Fig. 3; Table 1). At this stage we could detect low levels of expression of both USP and EcR in somatic cells at the tip of the ovary (not shown). In metamorphic ovaries, these cells form the anterior oviduct (see Fig. 4D), though this cell type does not differentiate in paedogenetic ovaries. Follicle cell differentiation continued at 18 h after birth (not shown), and at 24 h we detected high levels of USP and EcR expression in all of the somatic cells of the ovary, including the follicle cells (Fig. 5C, F) and surrounding sheath (not shown). At this stage, the pre-oviduct cells were no longer apparent, and some follicles had already been released from the ovary into the hemocoel (Table 1).

USP and EcR were not expressed in the oocyte nucleus at any stage examined (not shown; but see Fig. 4D). Transient expression of both EcR and USP was seen in

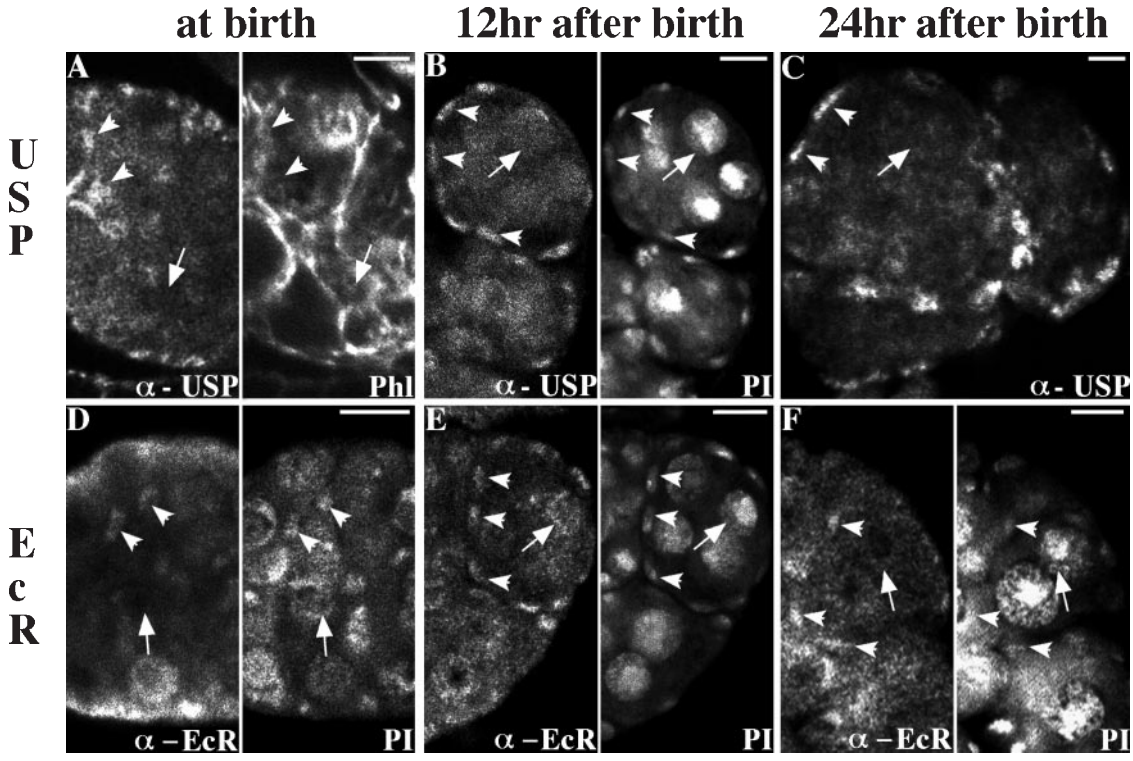

Fig. 5 Magnified views of USP (**A–C**) and EcR (**D–F**) expression in presumptive ovarian follicles during paedogenetic development in *H. pygmaea* (symbols and abbreviations as in Fig. 4). USP (**A**) and EcR (**D**) were strongly expressed at birth in the pre-follicular somatic cells (*arrowheads*), but not in the nurse cells (*arrow*). By 12 h (**B**, **E**) differentiated follicle cells (*arrowheads*) surrounded maturing follicles and expressed EcR (**E**) and high levels of USP (**B**). At this stage, transient expression of USP and EcR was seen in the nurse cells (*arrow*). By 24 h (**C**, **F**), the patterns of USP (**C**) and EcR (**F**) had not changed substantially as follicles were being released from the ovary. No appreciable USP (**C**) or EcR (**F**) was seen in the nurse cells at 24 h (*arrows*). All *scale bars* are 10 µm

the mesoderm-derived nurse cells at 12 h after birth (Fig. 5B, E), though not at earlier (Fig. 5A, D) or later (Fig. 5C, F) stages.

USP and EcR were expressed in the brain of paedogenetic larvae from at least 12 h before birth (not shown) until 24 h after birth (Fig. 6), and the expression patterns were indistinguishable from the early larval pattern seen in the brains of *H. pygmaea* metamorphs (not shown). Therefore, the up-regulation and expansion of USP and EcR expression in the ovary during paedogenetic development was not accompanied by similar changes in the expression patterns of these proteins in the brain. During the first 24 h after birth, the brain showed little growth (Fig. 6), in contrast to the substantial growth of the ovary during this period (Fig. 3).

USP and EcR localization during metamorphic development in *M. speyeri*

As in *H. pygmaea*, when *M. speyeri* larvae are reared on old food, the ovaries do not differentiate until the final

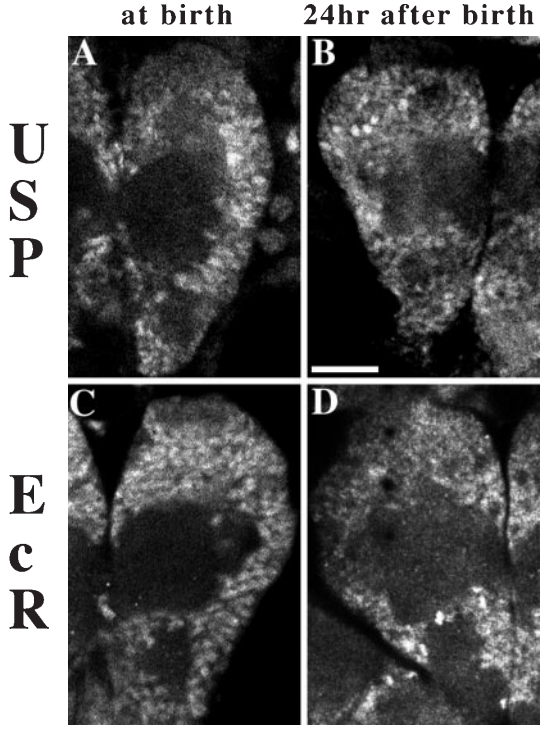

Fig. 6 USP (**A, B**) and EcR (**C, D**) expression in the brain during paedogenetic development in *H. pygmaea*. Both proteins were in a similar subset of cells in the brain at all stages examined [from pre-birth (not shown) through 24 h after birth]. Note the lack of change in the size of the brain. The *scale bar* is 20 µm and is the same in all figures

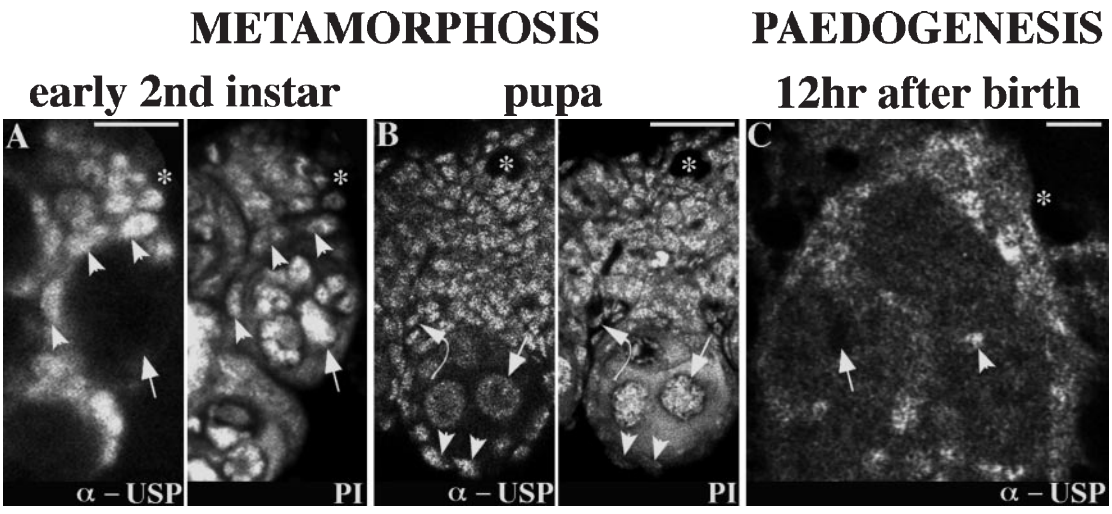

Fig. 7 USP expression during metamorphic (**A, B**) and paedogenetic (**C**) development in *M. speyeri* ovaries (symbols and abbreviations as in Fig. 4). Stages with significantly different USP patterns when compared with *H. pygmaea* are shown. **A** Early second instar metmaorph. High levels of USP were seen in the differentiating follicle cells (*arrowheads*) and pre-oviduct cells (*adjacent to the asterisk*), but not the nurse cells (*arrow*). **B** Pupa, one day after pupation. USP was seen in the follicle cells (*arrowheads*), sheath cells (*curved arrow*) and in nurse cells for the first time (*straight arrow*). Note the strong staining in the fully formed oviduct (*asterisk*). **C** Paedogenetic ovary, 12 h after birth. Pre-oviduct cells expressed high levels of USP. USP was also detected in the nascent follicle cells (*arrowhead*), but not in the nurse cells (*arrow*). All *scale bars* are 10 μm

larval instar (Fig. 3). During the first larval stage, the expression patterns of EcR and USP in *M. speyeri* (not shown) were essentially indistinguishable from that described above for *H. pygmaea* (Fig. 4A, B, E, F). About half of these old-food-reared *M. speyeri* larvae would molt to a second (final) instar larva, and proceed towards metamorphosis. The time from birth to ecdysis to the second instar varied widely among animals, presumably due to variability in food quality. Early second larval stage ovaries showed substantial levels of USP (Fig. 7 A) and low levels of EcR (not shown) expression in the pre-follicle cells and the pre-oviduct cells (as defined by Büning 1994). At the middle of the final larval stage (approximately 12 h later; see Table 2), the patterns of EcR and USP had not changed (not shown). At the pre-pupal stage, the differentiated follicle cells and sheath cells continued to express high levels of USP and EcR (not shown). This expression pattern was maintained through at least the first day of pupal development (Fig. 7B; not shown for EcR), as the follicles continued to grow. At around this stage, we detected low relative levels of USP expression in the nurse cells for the first time (Fig. 7B).

In summary, we detect low relative levels of USP and EcR in the ovary during early larval stages in *M. speyeri* metamorphs. Then, during the final larval stage, EcR and USP are up-regulated in the ovary as it begins to undergo its dramatic metamorphic changes.

USP and EcR localization during paedogenetic development in *M. speyeri*

When *M. speyeri* larvae are reared on fresh food, ovarian differentiation is morphologically apparent by about 12 h after the larvae hatch from their mother larva (Fig. 3; Table 1). We first detected USP and EcR expression at birth (not shown), in a pattern very similar to that in paedogenetic *H. pygmaea* larvae (Fig. 5A, D). At 6 h after birth, we detected low levels of USP and EcR expression in the pre-oviduct cells (not shown). However, since no oviduct is formed in these paedogenetic ovaries, we are unsure of the fate of these cells. By 12 h, USP (Fig. 7C) and EcR (not shown) continued to be expressed in these cells, and USP was generally expressed broadly throughout the somatic cells of the ovary (Fig. 7C). By 18 h after birth, follicles are clearly defined by the presence of USP-expressing follicle cells surrounding each nascent follicle (not shown). By 24 h, the follicles were just being released from the ovary (Table 1), and high levels of USP and EcR were still found in the follicle and interstitial somatic cells (not shown). Neither USP (Fig. 7C) nor EcR (not shown) was expressed in the oocyte or nurse cells at any stage examined.

USP and EcR were expressed in the brain of paedogenetic larvae from at least 12 h before birth until 24 h after birth, and the expression patterns were indistinguishable from the early larval pattern seen in the brains of *M. speyeri* metamorphs (not shown, but similar to Fig. 6). The up-regulation and expansion of USP and EcR expression in the ovary during paedogenetic development were not accompanied by similar changes in the expression patterns of these proteins in the brain. During the first 24 h after birth, the brain showed little growth (not shown), in contrast to the substantial growth of the ovary during this period (Fig. 3).

USP expression in *M. speyeri* and *H. pygmaea* metamorphs shifted to fresh food

When paedogenetic larval mothers of both species are placed on old food one day before they stop feeding,

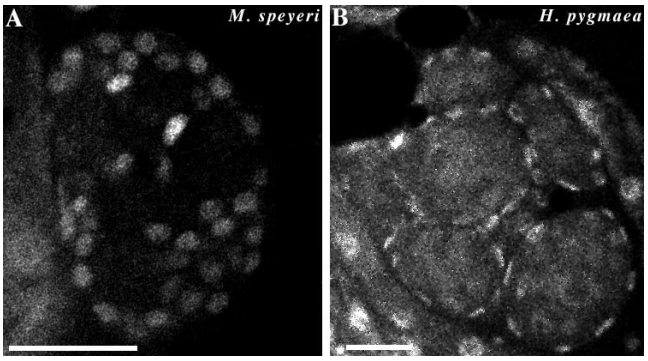

Fig. 8A,B Ovarian USP expression in metamorphs of both species, shifted to fresh food at birth, and dissected 24 h later. **A** *M. speyeri*. Note that the USP expression pattern is similar to *M. speyeri* paedogens 12–18 h after birth (compare with Fig. 7C). **B** *H. pygmaea*. Note that the USP expression pattern is similar to *H. pygmaea* paedogens 12–24 h after birth (compare with Fig. 5B, C). The *scale bars* are 20 μm

their daughters either develop towards metamorphosis or experience a developmental delay of several days before reproducing paedogenetically (Went 1975, 1979; J. Hodin, unpublished observations). However, if the daughter larvae are transferred to fresh food, they will reproduce paedogenetically after a delay of only several hours. To determine if this shift from metamorphic to paedogenetic development is also accompanied by up-regulation of USP, we transferred metamorphosis-destined daughter larvae of both species to fresh food at birth. In *M. speyeri*, USP was strongly up-regulated 24 h after transfer to fresh food (Fig. 8 A). The USP pattern and ovarian morphology were similar to *M. speyeri* paedogens 12–18 h after birth (compare with Fig. 7 C). A similar result was seen for *H. pygmaea* (Fig. 8B), though in this case, the USP pattern and ovarian morphology were similar to *H. pygmaea* paedogens 12–24 h after birth (compare with Fig. 5B, C).

Discussion

Major life history transformations such as metamorphosis are generally regulated by hormones. Thus one might hypothesize that evolutionary changes in the patterns of metamorphic events would involve alterations in hormone release, or in the tissue-specific responses to hormones. Indeed, this appears to hold for evolutionary changes in amphibian metamorphosis, where different steps in the thyroid hormone pathway are altered in independently-evolved neotenic (Frieden 1981; Yaoita and Brown 1990) and direct-developing (reviewed in Hanken et al. 1997) amphibians. Interestingly, while amphibians evolved metamorphosis independently of other deuterostomes such as echinoderms, thyroid hormones appear to be involved in sea urchin metamorphosis (Chino et al. 1994), and perhaps in evolutionary changes in metamorphosis in this group as well (Saito et al. 1998; Hodin et al. submitted). A similar pattern is seen in plants, where the independent evolution of the viviparous, mangrove phenotype in four taxa was in each case associated with reductions in levels of embryonic abscisic acid (Farnsworth and Farrant 1998), a plant hormone involved in desiccation tolerance of dormant seeds and responses to flooding and high salinity (reviewed in Kermode 1995). These findings suggest that the mechanisms of life history evolution may be biased towards alterations in hormones and/or the cellular hormonal response.

Paedogenesis in insects is characterized by the precocious differentiation of the ovary and subsequent parthenogenetic reproduction in a larval form. In the gall midges (Diptera: Cecidomyiidae), paedogenesis has evolved independently at least twice (Fig. 9 A). In *Drosophila melanogaster*, a dipteran with typical, metamorphic development, the two members of the ecdysone receptor complex necessary for the mediation of the molting and metamorphic actions of ecdysteroids, the Ecdysone Receptor (EcR) and Ultraspiracle (USP) (Yao et al. 1992, 1993), only appear in the ovary early in the final larval instar (Hodin and Riddiford, 1998). Moreover, the timing and pattern of expression of these two proteins in the differentiating cell types was shown to be necessary for the proper maturation of the ovary during metamorphosis.

Here we have extended these studies to two independently-evolved, paedogenetic gall midge species, *Heteropeza pygmaea* and *Mycophila speyeri*, to test the hypothesis that heterochronic changes in the ecdysone receptor complex can account for the evolution of the paedogenetic reproductive strategy. Previous studies had suggested that 20-hydroxyecdysone (20 E) regulates the onset of precocious ovarian differentiation in *H. pygmaea*, since the peak titer of 20 E in paedogenetic development occurs at the time of birth of the daughter larvae, shortly after which differentiation of the ovaries begins in the paedogenetic life cycle (Went et al. 1984). Furthermore, addition of 20 E in vitro (Went 1978) and in vivo (Treiblmayr et al. 1981) accelerates ovarian differentiation in *H. pygmaea*. Our studies show that the up-regulation of both EcR and USP in the ovaries of the two species occurs precociously (in the first larval stage, as early as 6 h after birth from the mother larva) in paedogenetic larvae. By contrast, these proteins normally accumulate in the ovaries of mid second (final) instar metamorphosing larvae, prior to the onset of somatic cell ovarian differentiation. The pattern of constitutive EcR and USP expression in the brain is similar in the paedogenetic and the metamorphosing forms. We interpret this difference as a heterochronic shift (change in the relative timing of developmental events) in the patterns of EcR and USP expression specifically in the ovary. These results are consistent with the idea that the temporal alteration in EcR/USP expression regulates the precocious differentiation of the ovaries in the paedogenetic life cycle of these two species.

A direct comparison of the cell type-specific patterns of ovarian expression of EcR and USP between *D. melanogaster* and the two gall midge species is some-

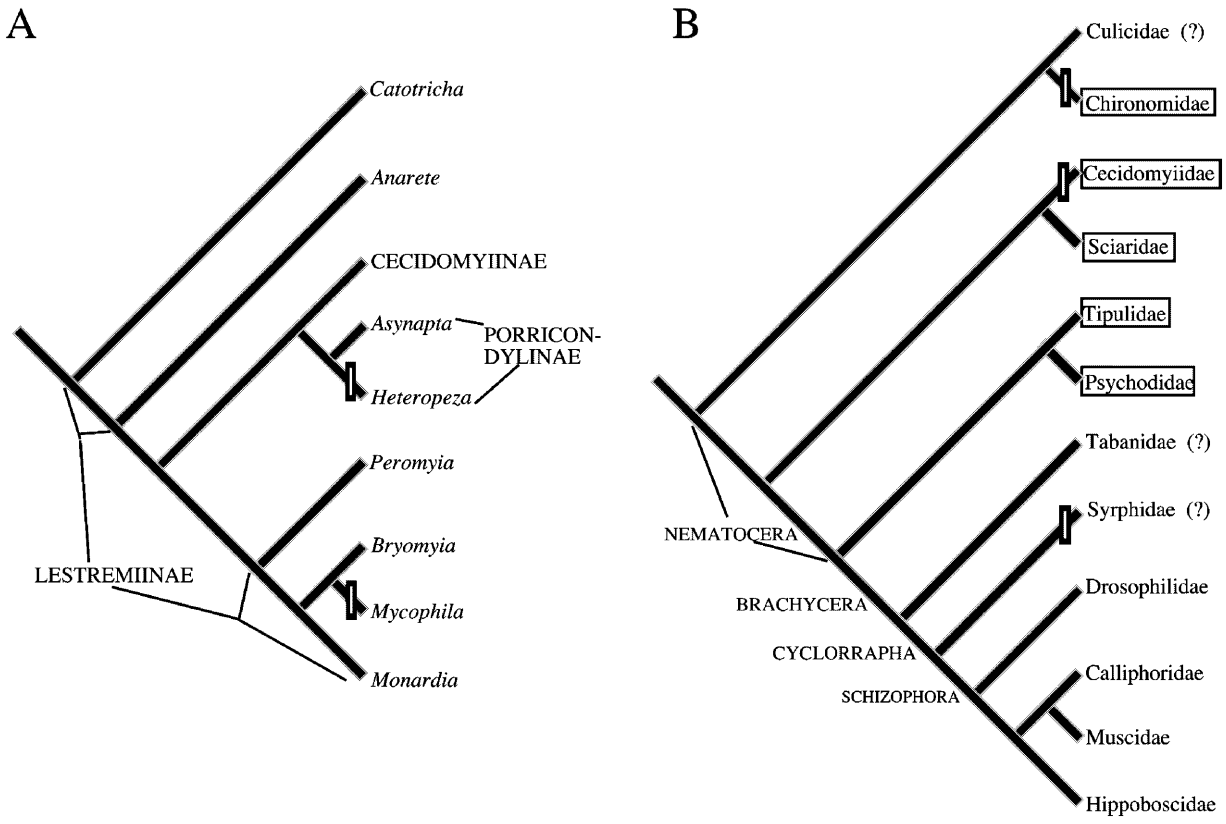

Fig. 9 **A** Phylogeny of the family Cecidomyiidae, after Jaschof's (1998) morphological analysis of lestremiine relationships using 41 informative characters. Taxa in *capital letters* are the three cecid sub-families: Lestremiinae, Porricondylinae and Cecidomyiinae. Note that according to this topology, the Lestremiinae are a paraphyletic group. Other taxa are representative genera from monophyletic clades. The *open bars* denote the paedogenetic genera. **B** Family level dipteran phylogeny after Yeates and Wiegmann (1999). Nematocera, Brachycera and Cyclorrapha are suborders (though Nematocera is paraphyletic). Schizophora is a series, a smaller taxonomic group above the family level. In families surrounded by a *box* (Tipulidae, Psychodidae, etc.), germ cell differentiation precedes ovarian somatic cell differentiation. In *non-boxed* families, the somatic cells differentiate first. The *question marks* denote families where this character has not been reported (see text). *Open bars* are families known to contain paedogenetic species (see text). Note that this is a highly simplified phylogeny, and that the Culicidae (mosquitoes), Tabanidae (deer and horse flies) and Syrphidae (flower flies) are only three of the many dipteran taxa in which the order of germ cell and somatic cell differentiation has not been reported

what problematic. Gall midges, even non-paedogenetic forms, have extremely derived patterns of ovarian differentiation. First, most species do not make ovarioles per se. The strings of egg chambers seen in some non-paedogenetic adult ovaries are the result of secondary fusion, not shared origin from a common germ line stem cell (Matuszewski 1968). Thus, structures such as the terminal filament cells are absent in these gall midges. Second, the dual germ cell-somatic cell origin of the nurse cells (see below) appears to be a feature unique to all gall midges. Finally, the one germ cell-cystocyte division and the formation of the nurse cell-oocyte complex take place during embryonic stages (reviewed in Büning 1994), while the ovaries are in a predifferentiative state, long before EcR and USP are expressed. In *D. melanogaster*, the comparable events of germ cell differentiation occur during pupal development (King et al. 1968).

Still, there are some clear similarities between ovarian expression of EcR and USP in *D. melanogaster* and the two gall midge species. In the reduced, metamorphic ovaries of *H. pygmaea* and *M. speyeri*, the three distinguishable, mature somatic cell types are the follicle cells, the oviduct cells and the sheath cells. All these cell types express EcR and USP as they differentiate. Similarly, at metamorphosis in *D. melanogaster*, EcR and USP expression correlates with differentiation of the follicle cells, oviduct and epithelial sheath cells (Hodin and Riddiford 1998). In paedogenetic gall midge ovaries of both species, the follicle cells are the predominant somatic cell type and express EcR and high levels of USP as they differentiate. By contrast, a group of pre-oviduct cells express only low levels of these two proteins during the early stage of ovarian differentiation, and have disappeared by the time that the follicles are ready to be released into the hemocoel. We do not know whether these cells undergo cell death or whether they contribute to another population of somatic cells. In ovaries of larvae destined for metamorphosis, high levels of EcR and USP appear in the pre-oviduct cells in the early to mid second larval instar at about the same time that these proteins appear in the follicle cells. Then by the prepupal stage, both the follicle cells and the oviduct have differentiated. Thus, high levels of EcR and USP correlate with oviduct

and follicle cell differentiation, whereas low levels may signal an alternative developmental pathway.

During metamorphic development in *M. speyeri* and *H. pygmaea*, both EcR and USP are present at low levels in the somatic cells of the ovary during the early larval stages, long before differentiation begins, which is presumably in response to high ecdysteroid levels that cause metamorphosis. Imaginal discs of early third (final) instar larvae of *D. melanogaster* also have low levels of both EcR and USP. At this stage, these proteins seem to be necessary for suppression of cell differentiation when little or no hormone is present, since differentiation of sensory neurons begins precociously in clones of wing-disc cells lacking functional USP when cultured in hormone-free conditions (Schubiger and Truman 2000). In this case, high levels of ecdysteroids acting through the EcR/USP complex are necessary for complete maturation of the adult structures. Similarly, the presence of EcR and USP at low levels in the early larval stage ovaries of the gall midge metamorphs, as well as in the pre-oviduct cells of the paedogenetic ovaries, may suppress differentiation until the ecdysteroid titer rises to high levels.

A second relevant finding from previous studies is that cell-type and/or temporal specificity of expression of different isoforms of USP in *Manduca* (Jindra et al. 1997) and of EcR in *Drosophila* and *Manduca* (Talbot et al. 1993; Truman et al. 1994) correlate with different types of responses (proliferation, differentiation, cell death, cuticle deposition etc.) to 20 E. In this study, we only examined antibodies which would recognize all isoforms of either EcR or USP (the isoform-specific EcR antibodies do not cross-react well with other species). It is certainly possible that differences between, for example, the pre-follicle cells (which differentiate early) and pre-oviduct cells (which do not) during paedogenetic ovarian differentiation could be explained by expression of different isoforms of EcR and/or USP in these different cell types. Still, how different cell types respond differently to ecdysteroids in insects remains largely a mystery, and is an active area of current research.

There is clearly a strong connection between larval nutrition, paedogenetic development and USP/EcR expression. When paedogenetic mothers of either species are placed on old food one day before the cessation of feeding, their daughters either undergo metamorphic development or are delayed in reproduction by several days. Such daughter larvae show only low levels of USP and EcR at birth, and expression is down-regulated during the first day after birth if they are kept on old food. The fact that differences in levels of USP and EcR in paedogenetic and metamorphic daughter larvae are already apparent at birth indicates that maternal nutrition influences the daughters' developmental trajectories, as has been previously suggested (Went 1977). Furthermore, if metamorphically-destined larvae of either species are shifted to fresh food at birth, USP is quickly up-regulated in their ovaries (with only a 6–12 h delay), and paedogenetic reproduction ensues. The connection between larval nutrition and ovary-specific induction of USP and EcR is not clear. Presumably there is some hemolymph-borne factor which is altered by larval nutrition and strongly influences ovarian morphogenesis. The nature of this factor, whether hormonal or otherwise, is currently unknown, and represents an important topic for future study.

Our results are consistent with the hypothesis that the precocious activation of ovarian differentiation in paedogenetic gall midges is accomplished by an ovary-specific ecdysone response, via up-regulation of the components of the ecdysone receptor specifically in the somatic cells of the ovary at an early stage. Furthermore, the patterns of precocious up-regulation of EcR and USP are remarkably similar in *H. pygmaea* and *M. speyeri*. According to the cecid phylogeny of Jaschof (1998), by far the most parsimonious explanation is that paedogenesis evolved independently in these two taxa (Fig. 9A). Without more resolution in the sub-family Porricondylinae, of which *H. pygmaea* is a member, and where paedogenesis is more widespread (reviewed in Matuszewski 1982), one cannot conclude if paedogenesis has evolved independently on more than these two occasions. Still, the apparent involvement of the EcR/USP system in the independent evolution of paedogenesis in both *H. pygmaea* and *M. speyeri* represents an example of parallel evolution (reviewed in Hodin 2000), and we discuss this point in further detail below.

EcR and USP are involved in the differentiation of the somatic cells of the dipteran ovary

In *D. melanogaster*, EcR and USP are coexpressed in all but one of the mesoderm-derived somatic cell types of the ovary, preceding or coincident with cell differentiation (Hodin and Riddiford 1998). The one exception is the terminal filament cells, the first cell type to differentiate in the *Drosophila* ovary [as well as in insect ovaries generally (Büning 1994); but not in gall midges (see below)], in which USP is expressed in the absence of EcR during differentiation. We never detected EcR nor USP expression in the germ cells at any time from the final larval stage through mid-metamorphosis (Hodin and Riddiford 1998). Germ-cell differentiation in *D. melanogaster* (defined here as the divisions of a primordial germ cell into the 16-cell oocyte-nurse-cell complex) does not begin until at least 12 h after the onset of metamorphosis, at which time many of the early differentiative events in the ovarian somatic cells are underway or complete (King et al. 1968; Hodin and Riddiford 1998). Thus it seems that the germ cell and somatic cell differentiation programs are under separate developmental control in *D. melanogaster*.

As is true in *D. melanogaster*, in the two gall midge species that we examined, EcR and USP expression was generally limited to the mesoderm-derived somatic cells of the ovary. We detected USP, and possibly EcR (not shown), in a subset of nurse cell nuclei of pupal ovaries

in both species, but by this stage, the events of ovarian differentiation (as opposed to oogenesis) are essentially complete. Both EcR and USP were transiently expressed in the nurse cells of *H. pygmaea* paedogenetic ovaries 12 h after birth. At this stage, nurse chamber formation is complete and follicle cell differentiation is underway. Perhaps EcR and USP play a role in the nurse cells in communicating to the differentiating follicle cells at this stage to help coordinate follicle cell differentiation. It is worth noting that in all gall midges examined, both paedogenetic and non-paedogenetic (Madhavan 1973; Mahowald and Stoiber 1974; Matuszewski 1978; Jazdowska-Zagrodzinska 1979; Schüpbach and Went 1983), the nurse cells have two embryonic origins: one nurse cell per chamber is a sister cell to the oocyte, while all the others (variable in number) are of somatic origin. These latter cells are incorporated into the oocyte-nurse chamber complex by cell fusion. It may be that the somatic nurse cells are the cells that express EcR and USP during these later stages.

As is generally true in *D. melanogaster* ovarian differentiation, EcR and USP appear to be coexpressed at all stages in gall midge ovaries. There are some stages where the pattern of ovarian USP expression appears to be more widespread than that of EcR, but we suspect that this may be an artifact of the efficacy of the two antibodies: the *Drosophila* USP antibody appears to cross-react with gall midge USP much more effectively than does the *Manduca* EcR antibody with gall midge EcR.

Our results with *D. melanogaster*, *H. pygmaea* and *M. speyeri* suggest that dipterans may share a common mechanism for differentiation of the ovarian somatic cells via the ecdysteroid response system. The functional data in *Drosophila* (Hodin and Riddiford 1998), combined with the expression pattern data for the three species at metamorphosis, the food shift experiments, and the observation that the ovarian somatic cells in gall midges also express EcR and USP during paedogenetic reproduction, suggest that these proteins play a fundamental role in regulating the timing of ovarian differentiation in dipteran insects. Still, it is possible that the similarities seen in gall midges and *Drosophila* are due to parallel evolution (independent evolution using the same developmental mechanism). To begin to distinguish between these two possibilities, one could examine EcR and USP expression during ovarian differentiation in dipterans such as horse flies (Tabanidae) and flower flies (Syrphidae), which are thought to have diverged from the main dipteran line before the evolution of the higher diptera (such as *Drosophila*; see Fig. 9B).

Dipteran evolution and developmental constraints: why is paedogenesis so rare?

Paedogenesis, defined broadly so as to include reproduction in the pupal as well as the larval stage, has evolved independently at least six times in insects: once in aphids (telescoped generations; see Dixon 1985), once in beetles (Barber 1913; Scott 1938), and at least four times in dipterans (including the two independent cases in gall midges; Wagner 1862; Felt 1911; Zavrel 1926; Wyatt 1961, 1963, 1964, 1967; Ibrahim and Gad 1975; Fig. 9). On the surface, it seems to be a highly adaptive strategy. When food resources are plentiful, the animals utilize that resource maximally without the risks and delays involved in metamorphosis. When food resources diminish, the animals proceed to metamorphosis to disperse and find a new food patch. So why has it evolved so infrequently? First of all, it is worth noting that paedogenesis may be more common than is generally realized, as detailed studies of larval development have only been carried out in a tiny percentage of extant insect species. Still, enough different species have been examined in sufficient detail to suggest that there may be some constraint on the evolution of paedogenesis outside of the taxa where it has been previously identified.

It is notable that all three of the dipteran families known to contain paedogenetic species are basal taxa (Fig. 9B). Interestingly, all of the Nematocera (a basal assemblage of dipterans including midges, mosquitoes and crane flies) that have been examined are characterized by early germ cell differentiation, often during embryogenesis (Büning 1994). These taxa (boxed in Fig. 9B) deviate from the typical insect pattern where, as in *Drosophila* and other higher flies (series Schizophora; see Fig. 9B), the somatic ovarian cell types differentiate before the germ cells. Outside the Diptera, early relative differentiation of the germ line has only been reported in the scale insects (Coccidae), a sister group to the aphids (Büning 1994). In paedogenetic aphids, somatic and germ cell differentiation occurs almost simultaneously (Büning 1994). In syrphids (flower flies), paedogenesis has been described for only one species, *Eristalis tenax*. To our knowledge, the order of germ cell and somatic cell differentiation has not been examined in this group (question mark in Fig. 9B). It seems that early germ cell differentiation arose early in dipteran evolution, and was lost in the lineage leading to the Schizophora (see Fig. 9B).

We propose the following hypothesis for the restricted evolutionary distribution of paedogenesis. The evolution of paedogenesis must be associated with several necessary pre-adaptations. One pre-adaptation must be parthenogenesis. In addition, both the somatic and the germ cell differentiation programs need to be precociously activated, and these two programs may be under separate developmental control (as evidenced by the evolutionary dissociability of these two processes, as well as the absence of EcR and USP expression in the differentiating germ cells of *D. melanogaster* and both paedogenetic gall midge species). There are undoubtedly other requirements as well. Therefore, assembling all the necessary pre-adaptations for larval reproduction may simply be a situation that arises infrequently in insect evolution. The early determination of germ cells in lower Diptera may predispose these taxa for paedogenesis.

Thus, perhaps it is not surprising to find that within the Cecidomyiidae, the mechanisms of paedogenesis

have evolved in parallel. Since the germ cells were already differentiating early in a hypothetical ancestral non-paedogenetic gall midge, the most important change necessary to evolve paedogenesis may have been the precocious activation of the ovarian somatic cell differentiation program. Since the timing of somatic cell differentiation appears to be regulated by the ecdysone system, different gall midges may be predisposed to evolve paedogenesis by a common mechanism. This may be appropriately seen as a developmental constraint (a "bias [in] the production of variant phenotypes caused by the...dynamics of the developmental system"; Maynard-Smith et al. 1985, page 266) on the evolution of paedogenesis. There may only be a limited set of possible ways to evolve this life cycle, and it may be essentially unavailable to many taxa due to the vagaries of evolutionary history (namely, which taxa have the appropriate pre-adaptations, such as parthenogenesis and early germ cell differentiation). Clearly a detailed examination of the known, phylogenetically disparate cases of paedogenesis is warranted, as it would address these hypotheses, and in so doing provide insight into the mechanisms underlying life history evolution.

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ERRATUM

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Parallel alterations in the timing of ovarian Ecdysone Receptor and Ultraspiracle expression characterize the independent evolution of larval reproduction in two species of gall midges (Diptera: Cecidomyiidae)

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Throughout the text and in the reference section, the name of the author of the phylogenetic analysis of the Cecidomyiidae was mis-spelled. His name is Mathias Jaschhof, not "Jaschof".

The authors regret the error.

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