Rethinking the ecdysteroid source during *Drosophila* pupal–adult development

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Abstract/Overview

Ecdysteroids, typified by 20-hydroxyecdysone (20E), are essential hormones for the development, reproduction and physiology of insects and other arthropods. For over half a century, the vinegar fly *Drosophila melanogaster* has been used as a model of ecdysteroid biology, with many aspects of the biosynthesis and regulation of ecdysteroids understood at the molecular level, particularly with respect to their secretion from the prothoracic gland (PG) cells of the ring gland, widely considered the dominant biosynthetic tissue during development. Discrete pulses of 20E orchestrate transitions during the *D. melanogaster* life cycle, the sources of which are generally well understood, apart from the large 20E pulse at the onset of pharate adult development, which has received little recent attention. As the source of this pharate adult pulse (PAP) is a curious blind spot in *Drosophila* endocrinology, we evaluate published biochemical and genetic data as they pertain to three hypotheses for the source of PAP 20E: the PG; an alternative biosynthetic tissue; or the recycling of stored 20E. Based on multiple lines of evidence, we contend the PAP cannot be derived from biosynthesis, with other data consistent with *D. melanogaster* being able to recycle ecdysteroids before and during metamorphosis. Further experimental work is required to test the ecdysteroid recycling hypothesis, which would establish fundamental knowledge of the function, regulation and evolution of metamorphic hormones in insects.

Introduction

Steroids are ubiquitous signalling molecules in animals and plants. In insects and other arthropods, polyhydroxylated steroids known as ecdysteroids [1] act as hormones that control development, reproduction, physiology, immunity and behaviour [2–7]. Ecdysteroids, of which 20-hydroxyecdysone (20E) is generally considered the major hormone, mediate their effects through various receptors, including EcR/Usp, DopEcR and DHR38 [6,8,9], and many aspects of their biosynthesis and metabolism are now well understood. This is particularly true in the model insect *Drosophila melanogaster*, through which a number of critical discoveries about ecdysteroids have been made (reviewed in [2,10,11]). However, as we contend here, there are key developmental aspects of ecdysteroid biology that have not yet been settled in this species and deserve closer experimental scrutiny.

Ecdysteroid biosynthesis

In *D. melanogaster*, as with all insects, the biosynthesis of ecdysteroids begins with dietary sterols [12–15]. Typically, the sterol considered in the literature is cholesterol (a C27 sterol), which ultimately yields 20E, although alternate fungal and plant sterols such as ergosterol and campesterol (C28 sterols) can be used to produce functional hormones such as makisterone A [16–18]. The sterol backbone is transformed by the sequential action of enzymes, including an oxygenase (Neverland/Nvd [19,20]), a dehydrogenase (Shroud/Sro [21]) and multiple cytochrome P450s (Spook/Spo,

57 Spookier/Spok, Phantom/Phm, Disembodied/Dib, Shadow/Sad and Shade/Shd) that
58 decorate it with up to four additional hydroxyl (-OH) groups [22–27] (Fig. 1A). Six of the
59 genes encoding these enzymes were originally identified via nine 'Halloween'
60 mutations, each of which give *Drosophila* embryos a characteristic ghostly pall
61 (reviewed in [10,28]). Subsequent research has added more candidate enzymes to the
62 biosynthesis pathway, including the P450 Cyp6u1 [29] and the glutathione S-
63 transferase Noppera-bo/Nobo [30–32], likely reflecting unresolved complexity in the
64 pathway and its evolutionary diversification. (The P450 Cyp6t3 has previously been
65 considered a Black Box candidate enzyme based on RNAi phenotypes [33], but recent
66 work using null alleles strongly suggests it does not have a role in ecdysteroid
67 biosynthesis [34].) Non-enzymatic proteins, such as regulators [35–37] and splicing
68 factors [38], have also been identified as having specific roles in this pathway. While
69 most ecdysteroid biosynthesis—in pre-adult life stages—is typically thought to be
70 confined to the prothoracic gland (PG) cells of the ring gland (RG), where the pathway
71 shuttles between enzymes located in the mitochondrial and endoplasmic reticulum, the
72 final hydroxylation at C20 occurs in various tissues that express Shd [22]. 20-
73 hydroxylation marks the final 'activation' of ecdysone (E) into 20E (or their C28/C29
74 equivalents), as the latter is generally considered the primary active ecdysteroid in
75 *Drosophila*, although there is evidence other ecdysteroids have unique signalling
76 functions [9,39–41]. In the rest of this paper, for simplicity, we will refer to all 20-
77 hydroxylated ecdysteroids as '20E' and their immediate precursors as 'E'.
78

Developmental functions of ecdysteroids

Ecdysteroids are key drivers of development across insects, including *Drosophila*. They are first required for egg development, which progresses through 14 stages, defined by the behaviours and characteristics of both the germline and somatic cells that make up the egg chamber [42,43]. Without ecdysteroids, the developing egg cannot proceed to the yolk-uptake stages, because the hormones are required in a variety of cell types in the ovary to induce the progression of egg chambers through these early developmental stages [44–46]. During embryogenesis, both the extraembryonic membranes and the germ band itself require ecdysteroid signalling to undergo germ band retraction and complete head involution and mouth hook development [47]. Later in embryogenesis, ecdysteroids are necessary for larval cuticle deposition, as demonstrated by the Halloween mutant phenotypes [28].

Upon hatching, juvenile insects cycle through alternating growth phases followed by moulting to the next nymphal or larval instar. In *Drosophila,* the moult to the 2nd and 3rd larval instars are preceded by a large pulse of ecdysteroids, which induce the development of instar-specific morphologies (such as the spiracles and head skeleton), apolysis of the previous instar's larval cuticle, and deposition of the new larval cuticle (reviewed in [48]). In the 3rd larval instar, there are a series of smaller pulses that prepare the larva for pupal and adult development [49]. The first pulse induces a transition known as critical weight, after which larvae can initiate metamorphosis even under adverse conditions like starvation [49,50]. The second small pulse initiates the

101 production of glue proteins in the salivary glands, which are used by the larvae to glue
102 itself to the substrate at pupariation [49,51]. The third pulse induces wandering
103 behaviour, where the larvae emerge from the food and search for a site to pupariate in
104 preparation for metamorphosis [49,52].

105

106 Following the three low-titre ecdysteroid pulses in the 3rd larval instar, a large 'prepupal
107 pulse' causes the larvae to cease wandering, become immobile, evert their spiracles,
108 and harden and tan their cuticle to form the puparium, initiating the prepupal stage
109 [49,53,54]. Further, in response to the prepupal pulse, the imaginal discs evert and
110 begin to deposit the pupal cuticle [55,56]. Approximately ten hours later, a relatively
111 small 'pupal pulse' induces the pupal moult, limb extension, head eversion, and the
112 degeneration of the PG cells [57,58].

113

114 A final, sizable 'pharate adult pulse' (PAP) occurs during the transition from pupa to
115 pharate adult, guiding the disintegration of larval structures while promoting the
116 differentiation of adult tissues. In the developing legs, wings, and notum, the PAP
117 induces the differentiation of sensory bristles, tarsal claws, and wing veins, and the
118 invagination of leg joints [59,60]. It also commences neuronal pruning and cell death of
119 the larval nervous system and the outgrowth and development of adult cells in the
120 central nervous system [61,62]. Indeed, it is this final 20E pulse that finishes off the
121 development of adult structures.

122

Known ecdysteroid sources

It is important to note the distinctions between ecdysteroid 'primary sources,' 'sites of conversion' and 'secondary sources,' as defined by Delbecque *et al.* [63]: primary sources synthesise ecdysteroids (typically prohormones, such as E) from sterols *de novo* and secrete them into the haemolymph; sites of conversion convert prohormones into active hormones (such as 20E, through the action of Shd), which then move throughout the body; and secondary sources secrete ecdysteroids that have been derived from a primary source and/or site of conversion, inactivated, stored and then released (ie. recycled) upon reception of an induction signal (Fig. 1A). The reversible inactivation of ecdysteroids can occur through conjugation (usually at C2, C3 and C22) with phosphate, glucose or fatty acids [64–66], although reversible C3 oxidation can also occur [67,68]. Well-known ecdysteroid sources are typically primary in nature, such as the PG, although some secondary sources have been studied in detail, such as the maternal ecdysteroid-phosphate conjugates hydrolysed during embryogenesis in the silkworm *Bombyx mori* [64,69]. Throughout the rest of this paper, with respect to ecdysteroid sources, 'primary' will be synonymous with 'biosynthetic,' and 'secondary' will be synonymous with 'recycling'.

With a couple of exceptions, ecdysteroid sources throughout the *D. melanogaster* life cycle are generally well understood. The epidermis is both a primary source and site of conversion during mid-embryogenesis before the formation of the RG, inferred from epidermal expression of Halloween genes [22,24–27]. A maternally derived secondary

145 source—likely ecdysteroid-acyl conjugates—appears to be present in the yolk at the
146 same time [70,71] and may play a role in some developmental processes in the embryo
147 [47], although the lethal phenotypes of zygotic Halloween mutants demonstrate this
148 maternal source is not sufficient for embryonic development, unlike in *B. mori* [72]. Once
149 the RG has formed, the PG cells are the dominant primary source of ecdysteroids
150 during late embryogenesis, as well as for the larval, prepupal and pupal pulses of 20E,
151 with various tissues acting as sites of conversion after hatching [22,52,73–75]. The
152 ovary acts as both a primary source and site of conversion in adult females [76–78], and
153 the accessory gland may have the same functions in adult males [79], although this has
154 yet to be definitively demonstrated.

155

156 The source of pharate adult 20E is unknown

157 Something that remains unclear is the source of the PAP, which to our knowledge has
158 never been examined in detail. Strangely, the PAP 20E titre is frequently assumed—
159 explicitly or implicitly—to be produced by the PG followed by immediate conversion,
160 particularly in recent reviews (eg. [80–84]), while older literature tends to be more critical
161 of this assumption (eg. [48,85–87]). We contend that the source of the PAP in *D.*
162 *melanogaster* is an unresolved question in insect endocrinology, and here we evaluate
163 three competing hypothetical sources of the PAP: the PG; another biosynthetic tissue;
164 or the recycling of 20E converted earlier in development (Fig. 1B).

165

The PG hypothesis

As the PG is the source of ecdysteroids for the larval, prepupal and pupal pulses, it is reasonable to hypothesise it is also the source of the PAP. In this hypothesis, the PG produces a large amount of E after pupation, which is then converted to 20E by Shd in one or more peripheral tissues, in a similar manner to what occurs earlier in development. Two significant problems for this hypothesis are that the PG cells begin to degrade at the start of metamorphosis, such that most are undergoing cell death at the peak of the PAP [86], and that cultured brain-RG complexes from early pharate adult animals secrete E at rates far below what would be required to produce the PAP through subsequent 20-hydroxylation [85,86]. As has been previously noted [50,51,53], these data strongly suggest that the PG cannot be the dominant source of ecdysteroids during the PAP.

The alternative primary source hypothesis

If the PG is not the source of E during the PAP, an attractive alternative hypothesis is that a different tissue is responsible for synthesising E after pupation, with conversion to 20E occurring either sequentially in the same tissue or in other tissues in the animal after secretion of E into the haemolymph. There is precedent for a non-PG primary source of ecdysteroids during embryogenesis in *D. melanogaster* (the epidermis; see above) and during metamorphosis in other insects, with the oenocytes and epidermis implicated in various species (reviewed in [63]), but an alternative primary source during

187 *D. melanogaster* metamorphosis has never—to our knowledge—been formally tested.
188 Consistent with this hypothesis, there is a small amount of (non-*shd*) Halloween gene
189 expression in non-PG larval tissues [88] and whole-body expression of most of these
190 genes is roughly sustained past pupation [89] (although the latter is also consistent with
191 continued ecdysteroid biosynthesis in the degenerating PG).

192

193 A substantial problem with the alternative primary source hypothesis, however, is the
194 very low level of *shd* expression [89] and ecdysone 20-monooxygenase activity [90]
195 detected after pupation, data consistent with the high ratio of E to 20E in this period
196 [16,54,91,92]. Notably, transgenic rescue experiments by Petryk *et al.* [22] do not
197 demonstrate a requirement for Shd after pupation. Taken together, these data strongly
198 suggest E is not being converted to 20E at the time of the PAP. If conversion to 20E
199 does not take place after pupation, the presence or absence of an alternative primary
200 source of ecdysteroids like E is inconsequential to the presence of 20E and suggests
201 PAP 20E must be produced another way.

202

203 There are also genetic data concerning the low-ecdysteroid mutants ecd^{1} and mld^{DTS-3}
204 that suggest the ecdysteroid biosynthetic pathway (in the PG or elsewhere) is
205 dispensable for the PAP.

206

207 *ecdysoneless* (*ecd*) encodes a splicing factor required for the correct splicing of *spok*
208 pre-mRNA (and also splicing of *EcR* mRNA and the correct expression of *nvd*, *phm* and
209 *dib*) [38] and may also be required for intracellular transport of ecdysteroid
210 intermediates in the PG [93]. ecd^{1} is a recessive, temperature-sensitive (TS), loss-of-
211 function allele of *ecd* that produces an ecdysteroid deficiency in larvae reared at a
212 restrictive temperature (RT) of 29 ºC [94,95] due to a reduction in E secretion from the
213 PG [96,97], although it also causes multiple other developmental defects [96–98], likely
214 due to aberrant splicing and/or regulation of a variety of mRNA transcripts [38].
215 Importantly, ecd^{1} animals moved from a permissive temperature (PT) to an RT at
216 pupariation have no qualitative differences in the shape, peak titre or relative timing of
217 the PAP compared to wild-type animals at the RT or ecd^{1} animals at the PT [96,99],
218 suggesting the aspects of E biosynthesis controlled by *ecd* are not required for the PAP.
219
220 *molting defective* (*mld*) encodes a transcription factor required for the expression of *nvd*
221 and *spok* in the PG [36,37] and loss-of-function mutant larvae have a low ecdysteroid
222 titre and fail to molt [35]. mld^{DTS-3} is a dominant, TS, likely hypomorphic loss-of-function
223 allele of *mld* (reported in [5,6,100] through a personal communication with P. Maroy and
224 also claimed in FlyBase [101]), heterozygotes of which have temperature-inducible
225 phenotypes very similar to those of *mld* null mutants [102–104]. Notably, mld^{DTS-3}/+
226 larvae shifted from a PT of 22 ºC to an RT of 29 ºC after the middle of the 3rd larval
227 instar have high viability to adulthood [102], suggesting *mld*'s function (ie. *nvd* and *spok*
228 expression in the PG) is not required at least during metamorphosis, or earlier

229 depending on the time scale over which the *mld*$^{DTS-3}$ allele affects ecdysteroid
230 biosynthesis at 29 ºC or the sensitivity of each developmental stage to the mutation's
231 *mld* hypomorphism.
232
233 Taken together, the data on *ecd*^{1} and *mld*$^{DTS-3}$ cited above suggest the biosynthesis and
234 secretion of E by the PG is not required for the PAP. However, some caveats could
235 affect our interpretations. While use of *ecd*^{1} to explore ecdysteroid functions has been
236 strongly discouraged due to pleiotropy [96–98], it undeniably disrupts E biosynthesis
237 both directly and indirectly [38,93]. On the other hand, *mld*$^{DTS-3}$ should be associated
238 with less pleiotropy due to the highly specific function of *mld* [37]; indeed, a small
239 proportion of *mld*$^{DTS-3}$/+ 3rd instar larvae injected with a wild-type RG successfully eclose
240 as adults at an RT [102], suggesting wild-type E secretion is sufficient to rescue
241 developmental progression in at least some animals. However, both mutations were
242 derived from EMS mutagenesis [94,104] and key studies did not use alternative alleles
243 to complement or otherwise validate the observed phenotypes [96,99,102], meaning
244 unknown secondary mutations (or balancer chromosome alleles for *mld*$^{DTS-3}$/+
245 heterozygotes) could be responsible for some unexpected phenotypes observed, such
246 as high pharate lethality in *mld*$^{DTS-3}$/+ animals shifted to an RT mid-metamorphosis, the
247 early temperature-insensitivity period of *mld*$^{DTS-3}$/+ animals shifted from a PT to an RT,
248 and the inability of 20E-feeding to rescue *mld*$^{DTS-3}$/+ pupae to adulthood at an RT [102].
249 Additionally, the time scale over which induction of *ecd* or *mld* loss-of-function leads to a
250 loss or reduction in the ecdysteroid biosynthetic capacity of the PG is unclear. Dissected

251 brain-RG complexes from *ecd*^{1} animals pre-incubated at an RT for 24 hr secrete
252 approximately 50% the level of E compared to those pre-incubated for 0 hr [97],
253 suggesting ecdysteroid secretion is not quickly abolished upon inactivation of *ecd*. Also,
254 while Garen *et al.* [94] found *ecd*^{1} larvae shifted to an RT 20–24 hrs pre-pupariation
255 have negligible levels of ecdysteroids at a time when control larvae produce the
256 prepupal peak, a very similar experiment with a 24 hr pre-pupariation PT-to-RT shift by
257 Belinski-Deutsch *et al.* [99] found no difference in the prepupal titre. To our knowledge,
258 no published information exists on ecdysteroid titres in *mld*$^{DTS-3}$ pharate adults.
259 Regardless of these uncertainties around the *ecd*^{1} and *mld*$^{DTS-3}$ alleles, given the lack of
260 ecdysone 20-monooxygenase activity after pupation discussed above, we argue our
261 interpretation of the data cited strongly point towards ecdysteroid biosynthesis not being
262 the proximal source of the ecdysteroids in the PAP.

263

264 **The recycling hypothesis**

265 Our third hypothesis for the source of the PAP is that it is derived from recycled
266 ecdysteroids: 20E synthesised earlier in development (from one or more of the
267 embryonic, larval, prepupal or pupal peaks; Fig. 1B) is inactivated, stored, reactivated
268 and then released from a secondary source after pupation. This hypothesis—previously
269 proposed by Schwartz *et al.* [87] and Dai & Gilbert [86]—is consistent with the data cited
270 above, as it does not rely on the presence of active Shd enzyme during the pupal–
271 pharate adult transition; likewise, it does not require E biosynthesis in the PG or any
272 other alternative primary source. There is precedent for ecdysteroid recycling in

273 *Drosophila* and other insects: as previously mentioned, *D. melanogaster* embryos
274 appear to contain maternal ecdysteroid-acyl conjugates [70,71], although there is only
275 indirect evidence that they influence embryogenesis [47]; eggs from multiple species of
276 Lepidoptera and Orthoptera contain maternal ecdysteroid-phosphate conjugates that
277 are hydrolysed during embryogenesis (reviewed in [64,105]); male *Anopheles gambiae*
278 mosquitoes gift an ecdysteroid-phosphate conjugate to females that is then
279 deconjugated to produce an active hormone that controls female mating behaviour
280 [106]; and in the dipteran *Sarcophaga peregrina*, conjugates of 20E are produced by
281 larvae and then hydrolysed during pupal development [107–109], although it is unclear
282 if this is the only source of 20E during this period.

283

284 Published experimental data suggest a secondary source of the *Drosophila* PAP is
285 highly plausible, but arguably stop short of supporting it conclusively. The high ratio of E
286 to 20E found after pupation [16,54,91] (but see [110] for conflicting data) in the absence
287 of Shd activity [90] is consistent with 20E being produced by the reactivation of inactive
288 ecdysteroids (such as conjugates or other metabolites) while the degenerating PG
289 produces a modest but significant amount of E that fails to be 20-hydroxylated.
290 Additionally, according to Pak & Gilbert [92] (but see [16] for conflicting data, which may
291 be due to ecdysteroid extraction and detection differences between studies), 70% of the
292 ecdysteroid content of white prepupae is comprised of high- and low-polarity
293 conjugates, the former of which at least can be hydrolysed *in vitro* to predominantly 20-

294 hydroxyecdysone and makisterone A, raising the possibility these are being stored for
295 use later in metamorphosis.
296
297 Another line of evidence that suggests PAP 20E might come from recycling is the ability
298 of exogenous 20E (supplemented in dietary media) to rescue a high proportion of *dib*
299 and *phm* Halloween null mutants to adulthood [111]. As ecdysteroid secretion in
300 discrete pulses is important for developmental transitions [73,112], reliance on
301 exogenous 20E in biosynthetic mutants means internal metabolic mechanisms can
302 likely control the ecdysteroid titre independently of intake. This is because: (1) it seems
303 unlikely that larvae would produce discrete 20E pulses by modulating their dietary
304 intake of supplemented media, as they are continuous feeders [113] and rely on
305 consistent nutrient intake to rapidly grow to meet developmental checkpoints (reviewed
306 in [114,115]); and (2) while active ecdysteroid titres can be reduced by catabolism and
307 excretion [112,116], increasing titres in post-feeding stages (ie. prepupae, pupae and
308 pharate adults) in the absence of biosynthesis likely relies on recycling, as suggested by
309 Schwartz *et al.* [87]. If such a mechanism exists in chemically rescued biosynthetic
310 mutants, it likely also exists in wild-type animals during developmental stages where
311 20E biosynthesis is not active, such as—hypothetically—in late pupae/pharate adults.
312 This is independently supported by the observation that feeding 20E to wild-type larvae
313 increases 20E pulse titres during both feeding and post-feeding stages—including the
314 PAP—compared to non-20E-fed larvae [87], which strongly suggests at least some of
315 the PAP titre is derived from 20E circulating during feeding stages.

316

317 Overall, our interpretation of all the data discussed above is that a recycling origin for
318 the PAP is the most plausible of the three hypotheses presented. However, there is a
319 clear need for further experimentation, using modern genetic and analytical chemical
320 methods, to formally test this hypothesis. If the recycling hypothesis is correct, many
321 details would need to be determined, including the inactive ecdysteroid metabolites and
322 enzymes involved, the tissue that acts as the secondary source, the mechanism by
323 which 20E is secreted, and the signalling pathways that control the recycling system.

324

325 Why is the source of the PAP important?

326 As the PAP controls many aspects of adult tissue development, understanding how this
327 pulse of ecdysteroids is produced and regulated is important for the use of *D.*
328 *melanogaster* as an endocrinological model for other insects, especially as the
329 molecular pathways that control the secretion of E from the PG (reviewed in [74,82])
330 and a hypothetical recycling system during metamorphosis could substantially differ. It
331 is also tempting to speculate that regulation of the PAP could be a method through
332 which *Drosophila* spp. and related insects adaptively specify the adult phenotype in
333 relation to the juvenile environment. The timing of the PAP acts as a 'switch' for
334 seasonal polyphenism in multiple butterfly species [117–121]: could a similar
335 mechanism exist in the pest *Drosophila suzukii*, which develops into winter and summer
336 adult morphs in response to environmental conditions [122]? Additionally, there is
337 substantial evidence that the proximal cause of pupal diapause—a phenomenon that

338 does not occur in *D. melanogaster* but does in other *Drosophila* spp. [123] and many
339 other dipterans [124–131]—is an absence of the PAP [132–136], suggesting the latter's
340 source is of fundamental importance to understand the regulation and evolution of
341 diapause in Diptera. Finally, in contrast with *D. melanogaster*, there is persuasive
342 evidence that the PAP is derived from biosynthesis in multiple lepidopteran species
343 [137–140], suggesting the source of the PAP may be evolutionarily labile and could
344 underpin some developmental differences between insect taxa.

345

346 Conclusion

347 In summary, while the source of 20E during the PAP is an unresolved question in
348 *Drosophila* biology, we contend that multiple lines of existing data, synthesised here for
349 the first time, support the hypothesis of a secondary source over a traditional primary
350 source such as the PG. This 20E recycling hypothesis, first proposed in the 1980s and
351 virtually neglected in recent years, deserves dedicated experimental testing, and the
352 knowledge gained from such efforts will greatly enrich our current understanding of
353 insect endocrinology.

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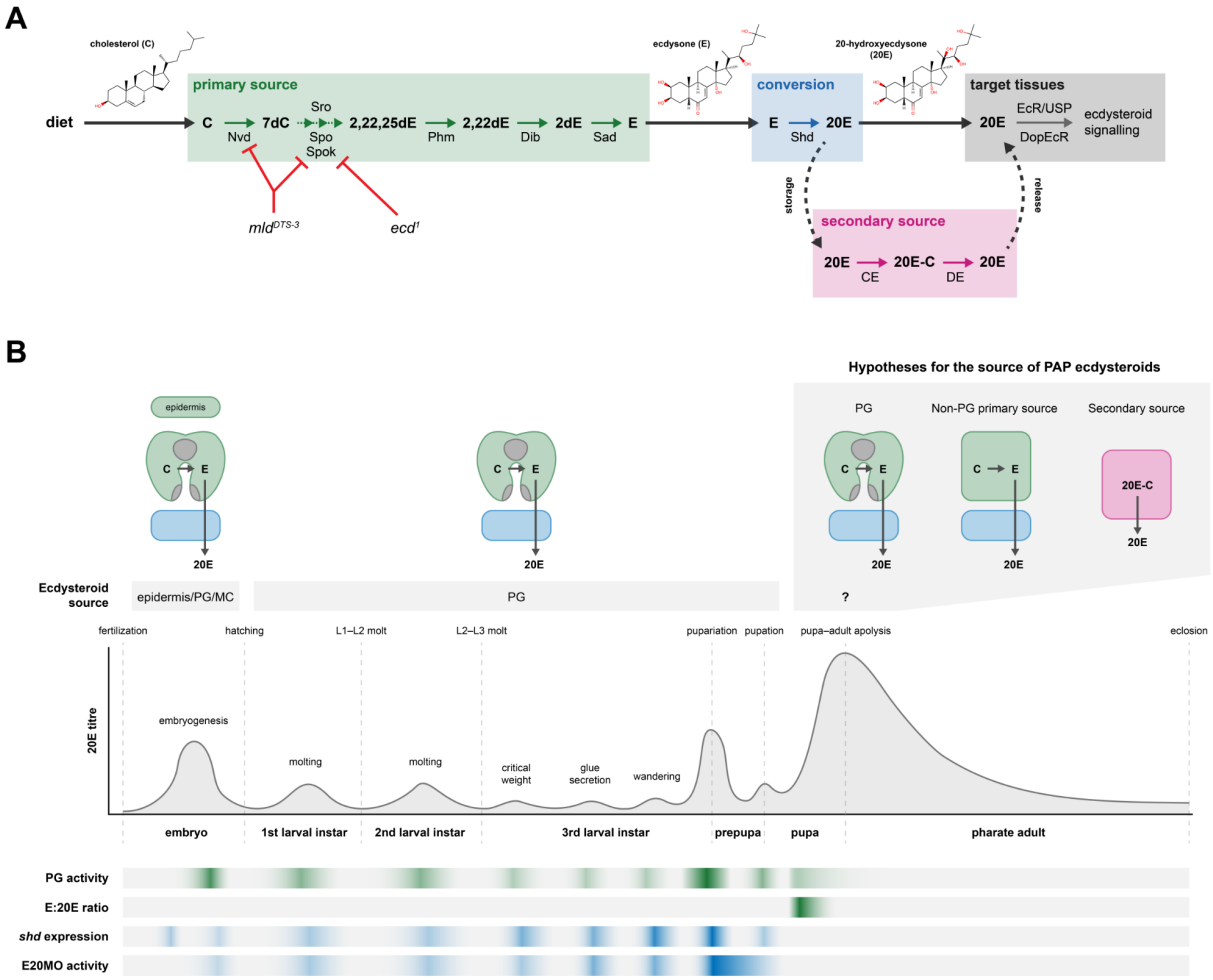

Figure 1. Three hypotheses for the source of 20E during *Drosophila* pharate adult development.

A) Schematic of ecdysteroid primary sources (green), sites of conversion (blue), hypothetical secondary sources (where the recycled hormone is 20E; pink) and ecdysteroid target tissues (grey). Only well-established biosynthetic enzymes are shown; the 'Black Box' is shown as multiple dashed arrows. Primary transcriptional effects of the *ecd*^{1} and *mld*$^{DTS-3}$ mutations are shown in red. C, cholesterol; 7dC, 7-dehydrocholesterol; 2,22,25dE, 2,22,25-deoxyecdysone/ketodiol; 2,22dE, 2,22-deoxyecdysone/ketotriol; 2dE, 2-deoxyecdysone; 20E-C; 20E conjugate; CE, conjugating enzyme; DE, deconjugating enzyme.

785 B) Ecdysteroid sources during *Drosophila* development, with major transitions and
786 developmental events labelled. Known and hypothetical sources (top), with the rough 20E titre
787 from embryo to eclosion (adapted from [48,49]; middle), and semi-quantitative depictions of PG
788 activity [85,86,89], *shd* expression [89], ecdysteroid 20-monooxygenase (E20MO) activity [90]
789 and E:20E ratio [16,54,91,92] (bottom). MC, maternal conjugates.