

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

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

Authors

Jack L Scanlan^{1*}, Charles Robin¹, Christen Mirth²

Affiliations

¹ School of BioSciences, The University of Melbourne, Parkville Campus, Melbourne,
Victoria, 3010, Australia

² School of Biological Sciences, Monash University, Melbourne, Victoria, 3800, Australia

* Corresponding author

Email: jacksca@gmail.com

17 **Abstract**

18 Ecdysteroids, typified by 20-hydroxyecdysone (20E), are essential hormones for the
19 development, reproduction and physiology of insects and other arthropods. For over
20 half a century, the vinegar fly *Drosophila melanogaster* (Ephydroidea: Diptera) has been
21 used as a model of ecdysteroid biology. Many aspects of the biosynthesis and
22 regulation of ecdysteroids in this species are understood at the molecular level,
23 particularly with respect to their secretion from the prothoracic gland (PG) cells of the
24 ring gland, widely considered the dominant biosynthetic tissue during development.
25 Discrete pulses of 20E orchestrate transitions during the *D. melanogaster* life cycle, the
26 sources of which are generally well understood, apart from the large 20E pulse at the
27 onset of pharate adult development, which has received little recent attention. As the
28 source of this pharate adult pulse (PAP) is a curious blind spot in *Drosophila*
29 endocrinology, we evaluate published biochemical and genetic data as they pertain to
30 three hypotheses for the source of PAP 20E: the PG; an alternative biosynthetic tissue;
31 or the recycling of stored 20E. Based on multiple lines of evidence, we contend the PAP
32 cannot be derived from biosynthesis, with other data consistent with *D. melanogaster*
33 able to recycle ecdysteroids before and during metamorphosis. Published data also
34 suggest the PAP is conserved across Diptera, with evidence for pupal–adult ecdysteroid
35 recycling occurring in other cyclorrhaphan flies. Further experimental work is required to
36 test the ecdysteroid recycling hypothesis, which would establish fundamental
37 knowledge of the function, regulation, and evolution of metamorphic hormones in
38 dipterans and other insects.

39 **Keywords**

- 40 ● Ecdysone
- 41 ● Hormone
- 42 ● Halloween
- 43 ● Metamorphosis
- 44 ● Conjugate
- 45 ● Endocrinology

46

47 **Abbreviations**

48 20E: 20-hydroxyecdysone

49 E: ecdysone

50 PAP: pharate adult pulse

51 PG: prothoracic gland

52 PT: permissive temperature

53 RG: ring gland

54 RNAi: RNA interference

55 RT: restrictive temperature

56 TS: temperature-sensitive

57

58 Declarations of interest: none

59

60 **Funding**

61 This work was supported by an Australian Government Research Training Program
62 (RTP) Scholarship.

63 **1. Introduction**

64 Steroids are ubiquitous signalling molecules in animals and plants. In insects and other
65 arthropods, polyhydroxylated steroids known as ecdysteroids (Goodwin et al., 1978) act
66 as hormones that control development, reproduction, physiology, immunity and
67 behaviour (Ishimoto et al., 2012; Ishimoto and Kitamoto, 2010; Nunes et al., 2021;
68 Petruccelli et al., 2020; Roy et al., 2018; Yamanaka et al., 2013). Ecdysteroids, of which
69 20-hydroxyecdysone (20E) is generally considered the major hormone, mediate their
70 effects through various receptors, including EcR/Usp, DopEcR and DHR38 (Baker et
71 al., 2003; Hill et al., 2013; Ishimoto et al., 2012), and many aspects of their biosynthesis
72 and metabolism are now well understood. This is particularly true in the model insect
73 *Drosophila melanogaster*, through which a number of critical discoveries about
74 ecdysteroids have been made (reviewed in Gilbert and Rewitz, 2009; Wieschaus and
75 Nüsslein-Volhard, 2016; Yamanaka et al., 2013). However, as we contend here, there
76 are key developmental aspects of ecdysteroid biology that have not yet been settled in
77 this species and deserve closer experimental scrutiny.

78

79 **1.1. Ecdysteroid biosynthesis**

80 In *D. melanogaster*, as with all insects, the biosynthesis of ecdysteroids begins with
81 dietary sterols (Carvalho et al., 2010; Clark and Block, 1959; Hobson, 1935a, 1935b).
82 Typically, the sterol considered in the literature is cholesterol (a C27 sterol), which
83 ultimately yields 20E, although alternate fungal and plant sterols such as ergosterol and
84 campesterol (C28 sterols) can be used to produce functional hormones such as

85 makisterone A (Blais et al., 2010; Feldlaufer et al., 1995; Lavrynenko et al., 2015). The
86 sterol backbone is transformed by the sequential action of enzymes, including an
87 oxygenase (Neverland/Nvd; Yoshiyama et al., 2006; Yoshiyama-Yanagawa et al.,
88 2011), a dehydrogenase (Shroud/Sro; Niwa et al., 2010) and multiple cytochrome
89 P450s (Spook/Spo, Spookier/Spok, Phantom/Phm, Disembodied/Dib, Shadow/Sad and
90 Shade/Shd) that decorate it with up to four additional hydroxyl (-OH) groups (Chavez et
91 al., 2000; Namiki et al., 2005; Ono et al., 2006; Petryk et al., 2003; Warren et al., 2004,
92 2002; Fig. 1A). Six of the genes encoding these enzymes were originally identified via
93 nine ‘Halloween’ mutations, each of which give *Drosophila* embryos a characteristic
94 ghostly pall (reviewed in Rewitz et al., 2006a; Wieschaus and Nüsslein-Volhard, 2016).
95 Subsequent research has added more candidate enzymes to the biosynthesis pathway,
96 including the P450 Cyp6u1 (Christesen et al., 2017) and the glutathione S-transferase
97 Noppera-bo/Nobo (Chanut-Delalande et al., 2014; Enya et al., 2014; Škerlová et al.,
98 2020), likely reflecting unresolved complexity in the pathway and its evolutionary
99 diversification. The P450 Cyp6t3 has previously been considered a Black Box candidate
100 enzyme based on RNAi phenotypes (Ou et al., 2011), but recent work using null alleles
101 strongly suggests it does not have a role in ecdysteroid biosynthesis (Shimell and
102 O’Connor, 2022). Non-enzymatic proteins, such as regulators (Danielsen et al., 2014;
103 Neubueser et al., 2005; Uryu et al., 2018) and splicing factors (Claudius et al., 2014),
104 have also been identified as having specific roles in this pathway. While most
105 ecdysteroid biosynthesis—in pre-adult life stages—is typically thought to be confined to
106 the prothoracic gland (PG) cells of the ring gland (RG), where the pathway shuttles

107 between enzymes located in the mitochondrial and endoplasmic reticulum, the final
108 hydroxylation at C20 occurs in various tissues that express Shd (Petryk et al., 2003).
109 20-hydroxylation marks the final 'activation' of ecdysone (E) into 20E (or their C28/C29
110 equivalents), as the latter is generally considered the primary active ecdysteroid in
111 *Drosophila*, although there is evidence other ecdysteroids have unique signalling
112 functions (Baker et al., 2003; Ono, 2014; Sommé-Martin et al., 1988a, 1990). In the rest
113 of this paper, for simplicity, we will refer to all 20-hydroxylated ecdysteroids (including
114 20-hydroxyecdysone, makisterone A and 24(28)-dehydromakisterone A; Lavrynenko et
115 al., 2015) as '20E' and their immediate precursors as 'E', except when talking about
116 specific conjugates or metabolites.

117

118 Ecdysteroid biosynthesis is also regulated by other endocrine factors throughout the
119 *Drosophila* life cycle. Juvenile hormone (JH), a family of sesquiterpenoids, is
120 synthesised by the corpus allatum cells of the RG and generally antagonises
121 ecdysteroidogenesis in the PG cells (Liu et al., 2017; Richard and Gilbert, 1991; Zhang
122 et al., 2018). In addition, a variety of neuropeptides and peptide hormones are known to
123 regulate E synthesis in the PG cells. Prothoracicotropic hormone (PTTH) is a neuronally
124 produced peptide that positively regulates Halloween gene transcription via the Tor
125 pathway (McBrayer et al., 2007; Rewitz et al., 2009). The *Drosophila* insulin-like
126 peptides (Dilps) both positively and negatively regulate ecdysteroidogenesis at different
127 points throughout larval development, largely through the Insulin Receptor (reviewed in
128 Kannangara et al., 2021). At least eight other peptide hormones are known to regulate

129 E synthesis in the PG cells, although their effects have been less well defined (reviewed
130 in Kannangara et al., 2021).

131

132 **1.2. Developmental functions of ecdysteroids**

133 Ecdysteroids are key drivers of development across insects, including *Drosophila*. They
134 are first required for egg development, which progresses through 14 stages, defined by
135 the behaviours and characteristics of both the germline and somatic cells that make up
136 the egg chamber (Cummings and King, 1969; King et al., 1956). Without ecdysteroids,
137 the developing egg cannot proceed to the yolk-uptake stages, because the hormones
138 are required in a variety of cell types in the ovary to induce the progression of egg
139 chambers through these early developmental stages (Bownes, 1989; Buszczak et al.,
140 1999; Wilson, 1982). During embryogenesis, both the extraembryonic membranes and
141 the germ band itself require ecdysteroid signalling to undergo germ band retraction and
142 complete head involution and mouth hook development (Kozlova and Thummel, 2003).
143 Later in embryogenesis, ecdysteroids are necessary for larval cuticle deposition, as
144 demonstrated by the Halloween mutant phenotypes (Rewitz et al., 2006a).

145

146 Upon hatching, juvenile insects cycle through alternating growth phases followed by
147 molting to the next nymphal or larval instar. In *Drosophila*, the molts to the 2nd and 3rd
148 larval instars are preceded by large pulses of ecdysteroids, which induce the
149 development of instar-specific morphologies (such as the spiracles and head skeleton),
150 apolysis of the previous instar's larval cuticle, and deposition of the new larval cuticle

151 (reviewed in Riddiford, 1993). In the 3rd larval instar, there are a series of smaller pulses
152 that prepare the larva for pupal and adult development (Warren et al., 2006). The first
153 pulse induces a transition known as critical weight, after which larvae can initiate
154 metamorphosis even under adverse conditions like starvation (Koyama et al., 2014;
155 Warren et al., 2006). The second small pulse initiates the production of glue proteins in
156 the salivary glands, which are used by the larvae to glue itself to the substrate at
157 pupariation (Hansson and Lambertsson, 1989; Warren et al., 2006). The third pulse
158 induces wandering behaviour, where the larvae emerge from the food and search for a
159 site to pupariate in preparation for metamorphosis (Parvy et al., 2005; Warren et al.,
160 2006).

161

162 Following the three low-titre ecdysteroid pulses in the 3rd larval instar, a large ‘prepupal
163 pulse’ causes the larvae to cease wandering, become immobile, evert their spiracles,
164 and harden and tan their cuticle to form the puparium, initiating the prepupal stage
165 (Berreur et al., 1979; Handler, 1982; Warren et al., 2006). Further, in response to the
166 prepupal pulse, the imaginal discs evert and begin to deposit the pupal cuticle (Fristrom
167 and Liebrich, 1986; Fristrom et al., 1973). Approximately 10 hours later, a relatively
168 small ‘pupal pulse’ induces the pupal molt, limb extension, head eversion, and the
169 degeneration of the PG cells (D’Avino and Thummel, 1998; Lam et al., 1999).

170

171 A final, sizable ‘pharate adult pulse’ (PAP) occurs during the transition from pupa to
172 pharate adult (including pupal–adult apolysis), guiding the disintegration of larval

173 structures while promoting the differentiation of adult tissues. In the developing legs,
174 wings, and notum, the PAP induces the differentiation of sensory bristles, tarsal claws,
175 and wing veins, and the invagination of leg joints (Fristrom, 1993; Mirth, 2005). It also
176 commences neuronal pruning and cell death of the larval nervous system and the
177 outgrowth and development of adult cells in the central nervous system (Robinow et al.,
178 1993; Truman et al., 1994). Indeed, it is this final 20E pulse that finishes off the
179 development of adult structures.

180

181 **2. Known ecdysteroid sources in *Drosophila***

182 It is important to note the distinctions between ecdysteroid ‘primary sources,’ ‘sites of
183 conversion’ and ‘secondary sources,’ as defined by Delbecque et al. (1990): primary
184 sources synthesise ecdysteroids (typically prohormones, such as E) from sterols *de*
185 *novo* and secrete them into the haemolymph; sites of conversion convert prohormones
186 into active hormones (such as 20E, through the action of Shd), which then move
187 throughout the body; and secondary sources secrete ecdysteroids that have been
188 derived from a primary source and/or site of conversion, inactivated, stored and then
189 released (ie. recycled) upon reception of an induction signal (Fig. 1A). The reversible
190 inactivation of ecdysteroids can occur through conjugation (usually at C2, C3 and C22)
191 with phosphate, glucose, acetate or fatty acids (Rharrabe et al., 2007; Sonobe and Ito,
192 2009; Thompson et al., 1987), although reversible C3 oxidation can also occur (Sun et
193 al., 2016; Webb et al., 1995). Well-known ecdysteroid sources are typically primary in
194 nature, such as the PG, although some secondary sources have been studied in detail,

195 such as the maternal ecdysteroid-phosphate conjugates hydrolysed during
196 embryogenesis in the silkworm *Bombyx mori* (Bombycoidea: Lepidoptera; Fujinaga et
197 al., 2020; Sonobe and Ito, 2009). Throughout the rest of this paper, with respect to
198 ecdysteroid sources, 'primary' will be synonymous with 'biosynthetic,' and 'secondary'
199 will be synonymous with 'recycling'.

200

201 Ecdysteroid sources throughout most of the *D. melanogaster* life cycle are generally
202 well understood, with a few exceptions. In adult males, the accessory gland may be a
203 primary source and site of conversion (Hentze et al., 2013), but this has yet to be
204 confirmed biochemically. In adult females, the ovary is generally considered the
205 dominant primary source and site of conversion, with biochemical evidence of
206 ecdysteroid secretion from this tissue (Rubenstein et al., 1982; Schwartz et al., 1989,
207 1985; Tu et al., 2002; but see Bownes et al., 1984; Handler, 1982; Schwedes and
208 Carney, 2012; Warren et al., 1996) and genetic evidence that ecdysteroid biosynthesis
209 and conversion in the ovary is required for normal female fertility (Ameku et al., 2017;
210 Ameku and Niwa, 2016; Domanitskaya et al., 2014; Knapp and Sun, 2017; Ono et al.,
211 2006; Petryk et al., 2003).

212

213 There is also some evidence that ovary-derived ecdysteroid conjugates are loaded into
214 oocytes to act as a secondary source during early embryogenesis. *Drosophila* yolk
215 proteins release esterase-sensitive ecdysteroid conjugates when degraded (Bownes et
216 al., 1988), suggesting these stored ecdysteroids are fatty acyl conjugates (Bownes,

217 1992); this is consistent with the detection of highly apolar ecdysteroids in ovaries (Grau
218 et al., 1995; Grau and Lafont, 1994a). Ovaries also may produce and/or secrete highly
219 polar ecdysteroids (Dübendorfer and Maróy, 1986), some of which are likely 22-
220 phosphates (Grau et al., 1995; Pis et al., 1995), although, to our knowledge, there is no
221 evidence these are loaded into oocytes.

222

223 The ecdysteroid content of whole early embryos is poorly studied: older studies suggest
224 that early embryos contain an unidentified ecdysteroid less polar than ecdysone that
225 disappears by mid-embryogenesis (Maróy et al., 1988) and that eggs contain highly
226 apolar conjugates that are likely 22-fatty acyl esters (Grau and Lafont, 1994b).

227 However, a more recent study did not detect ecdysteroid metabolites during embryonic
228 nor post-embryonic development (Lavrynenko et al., 2015), which conflicts with the
229 previously cited studies, possibly due to technical differences in methodology. The
230 developmental importance of a possible maternal secondary source in embryos is also
231 unclear: females temporarily unable to synthesise ecdysteroids produce offspring with
232 high rates of mid-embryonic developmental defects, suggesting maternally derived
233 ecdysteroids may partially control early to mid-embryogenesis (Kozlova and Thummel,
234 2003). Overall, data on such a maternal secondary source is mixed, and further,
235 focused experiments are required to test its presence/absence and molecular
236 characteristics. Regardless, the lethal phenotypes of zygotic Halloween mutants in *D.*
237 *melanogaster* demonstrate a maternal source cannot be sufficient for embryonic

238 development, unlike in *B. mori*, where Halloween mutants with severe defects in
239 ecdysteroid biosynthesis can complete embryogenesis (Niwa and Niwa, 2014).

240

241 The embryonic epidermis is both a primary source and site of ecdysteroid conversion
242 during mid-embryogenesis before the formation of the RG, inferred from epidermal
243 expression of Halloween genes (Chavez et al., 2000; Namiki et al., 2005; Ono et al.,
244 2006; Petryk et al., 2003; Warren et al., 2002). After the formation of the RG, the PG
245 cells are the dominant primary source of ecdysteroids through late embryogenesis to
246 the larval, prepupal and pupal pulses of 20E, with various tissues acting as sites of
247 conversion after hatching (Akagi et al., 2016; Buhler et al., 2018; Kannangara et al.,
248 2021; Parvy et al., 2005; Petryk et al., 2003).

249

250 **3. The source of pharate adult 20E is unknown**

251 Something that remains unclear is the source of the PAP, which to our knowledge has
252 never been examined in detail. Strangely, the PAP 20E titre is frequently assumed—
253 explicitly or implicitly—to be produced by the PG followed by immediate conversion,
254 particularly in recent reviews (eg. Kamiyama and Niwa, 2022; Lee and Park, 2021;
255 Malita and Rewitz, 2021; Pan et al., 2021; Xu et al., 2020), while older literature tends to
256 be more critical of this assumption (eg. Dai and Gilbert, 1991; Redfern, 1983; Riddiford,
257 1993; Schwartz et al., 1984). We contend that the source of the PAP in *D. melanogaster*
258 is an unresolved question in insect endocrinology, and here we evaluate three

259 competing hypothetical sources of the PAP: the PG; another biosynthetic tissue; or the
260 recycling of 20E converted earlier in development (Fig. 1B).

261

262 **3.1. The PG hypothesis**

263 As the PG is the source of ecdysteroids for the larval, prepupal and pupal pulses, it is
264 reasonable to hypothesise it is also the source of the PAP. In this hypothesis, the PG
265 produces a large amount of E after pupation, which is then converted to 20E by Shd in
266 one or more peripheral tissues, in a similar manner to what occurs earlier in
267 development. Two significant problems for this hypothesis are that the PG cells begin to
268 degrade at the start of metamorphosis, such that most are undergoing cell death at the
269 peak of the PAP (Dai and Gilbert, 1991), and that cultured brain-RG complexes from
270 early pharate adult animals secrete E at rates far below what would be required to
271 produce the PAP through subsequent 20-hydroxylation (Dai and Gilbert, 1991; Redfern,
272 1983). As has been previously noted (Dai and Gilbert, 1991; Redfern, 1983; Riddiford,
273 1993), these data strongly suggest that the PG cannot be the dominant source of
274 ecdysteroids during the PAP.

275

276 **3.2. The alternative primary source hypothesis**

277 If the PG is not the source of E during the PAP, an attractive alternative hypothesis is
278 that a different tissue is responsible for synthesising E after pupation, with conversion to
279 20E occurring either sequentially in the same tissue or in other tissues in the animal

280 after secretion of E into the haemolymph. There is precedent for a non-PG primary
281 source of ecdysteroids during embryogenesis in *D. melanogaster* (the epidermis; see
282 above) and during metamorphosis in other insects, with the oenocytes and epidermis
283 implicated in various species (reviewed in Delbecque et al., 1990). An alternative
284 primary source during *D. melanogaster* metamorphosis has never—to our knowledge—
285 been formally tested with either biochemical analyses of the presence of Halloween
286 enzymes in non-PG tissues during the PAP, nor with *ex vivo* ecdysteroid secretion
287 experiments like those performed in other insect species (Delbecque et al., 1990;
288 Jenkins et al., 1992; Telang et al., 2007).

289

290 Despite this absence of supportive biochemical data, there is a small amount of (non-
291 *shd*) Halloween gene expression in non-PG larval tissues (Leader et al., 2018) and
292 whole-body expression of most of these genes is roughly sustained past pupation
293 (Graveley et al., 2011). These data are consistent with this hypothesis, although the
294 latter is also consistent with continued ecdysteroid biosynthesis in the degenerating PG.
295 However, there are multiple sources of biochemical and genetic data that strongly
296 conflict with this hypothesis for the source of the PAP, which we outline below.

297

298 The first substantial problem with the alternative primary source hypothesis is the very
299 low level of *shd* expression (Graveley et al., 2011) and Shd/ecdysone 20-
300 monooxygenase activity (Mitchell and Smith, 1988) detected after pupation, data
301 consistent with the high ratio of E to 20E in this period (An et al., 2017; Handler, 1982;

302 Lavrynenko et al., 2015; Pak and Gilbert, 1987). Notably, transgenic rescue
303 experiments by Petryk et al. (2003) do not demonstrate a requirement for Shd after
304 pupation. Taken together, these data strongly suggest E is not being converted to 20E
305 at the time of the PAP. If conversion to 20E does not take place after pupation, the
306 presence or absence of an alternative primary source of ecdysteroids like E is
307 inconsequential to the presence of 20E and suggests PAP 20E must be produced
308 another way.

309

310 There are also genetic data detailed below concerning the low-ecdysteroid mutants
311 *ecd¹* and *mld^{DTS-3}* that suggest the ecdysteroid biosynthetic pathway (in the PG or
312 elsewhere) is dispensable for the PAP.

313

314 *ecdysoneless (ecd)* encodes a splicing factor required for the correct splicing of *spok*
315 pre-mRNA (and also splicing of EcR mRNA and the correct expression of *nvd*, *phm* and
316 *dib*; Claudius et al., 2014) and may also be required for intracellular transport of
317 ecdysteroid intermediates in the PG (Warren et al., 1996). *ecd¹* is a recessive,
318 temperature-sensitive (TS), loss-of-function allele of *ecd* that produces an ecdysteroid
319 deficiency in larvae reared at a restrictive temperature (RT) of 29 °C (Garen et al., 1977;
320 Sommé-Martin et al., 1988b) due to a reduction in E secretion from the PG (Henrich et
321 al., 1987; Redfern and Bownes, 1983), although it also causes multiple other
322 developmental defects (Gaziova et al., 2004; Henrich et al., 1987; Redfern and Bownes,
323 1983), likely due to aberrant splicing and/or regulation of a variety of mRNA transcripts

324 (Claudius et al., 2014). Importantly, *ecd¹* animals moved from a permissive temperature
325 (PT) to an RT at pupariation have no qualitative differences in the shape, peak titre or
326 relative timing of the PAP compared to wild-type animals at the RT or *ecd¹* animals at
327 the PT (Belinski-Deutsch et al., 1983; Redfern and Bownes, 1983), suggesting the
328 aspects of E biosynthesis controlled by *ecd* are not required for the PAP.

329

330 *molting defective (mld)* encodes a transcription factor required for the expression of *nvd*
331 and *spok* in the PG (Danielsen et al., 2014; Uryu et al., 2018) and loss-of-function
332 mutant larvae have a low ecdysteroid titre and fail to molt (Neubueser et al., 2005).

333 *mld^{DTS-3}* is a dominant, TS, likely hypomorphic loss-of-function allele of *mld* (reported in
334 Ishimoto et al., 2012; Ishimoto and Kitamoto, 2010; Simon et al., 2003 through a
335 personal communication with P. Maroy and also claimed in FlyBase; Thurmond et al.,
336 2019), heterozygotes of which have temperature-inducible phenotypes very similar to
337 those of *mld* null mutants (Holden and Suzuki, 1973; Holden et al., 1985; Walker et al.,
338 1987). Notably, *mld^{DTS-3/+}* larvae shifted from a PT of 22 °C to an RT of 29 °C after the
339 middle of the 3rd larval instar have high viability to adulthood (Holden et al., 1985),
340 suggesting *mld*'s function (ie. *nvd* and *spok* expression in the PG) is not required at
341 least during metamorphosis, or earlier depending on the time scale over which the
342 *mld^{DTS-3}* allele affects ecdysteroid biosynthesis at 29 °C or the sensitivity of each
343 developmental stage to the mutation's *mld* hypomorphism.

344

345 Taken together, the data on *ecd^l* and *mld^{DTS-3}* cited above suggest the biosynthesis and
346 secretion of E by the PG is not required for the PAP. However, some caveats could
347 affect our interpretations. While use of *ecd^l* to explore ecdysteroid functions has been
348 strongly discouraged due to pleiotropy (Gaziova et al., 2004; Henrich et al., 1987;
349 Redfern and Bownes, 1983), it undeniably disrupts E biosynthesis both directly and
350 indirectly (Claudius et al., 2014; Warren et al., 1996). On the other hand, *mld^{DTS-3}*
351 should be associated with less pleiotropy due to the highly specific function of *mld* (Uryu
352 et al., 2018); indeed, a small proportion of *mld^{DTS-3/+}* 3rd instar larvae injected with a
353 wild-type RG successfully eclose as adults at an RT (Holden et al., 1985), suggesting
354 wild-type E secretion is sufficient to rescue developmental progression in at least some
355 animals. However, both mutations were derived from EMS mutagenesis (Garen et al.,
356 1977; Holden and Suzuki, 1973) and key studies did not use alternative alleles to
357 complement or otherwise validate the observed phenotypes (Belinski-Deutsch et al.,
358 1983; Holden et al., 1985; Redfern and Bownes, 1983), meaning unknown secondary
359 mutations (or balancer chromosome alleles for *mld^{DTS-3/+}* heterozygotes) could be
360 responsible for some unexpected phenotypes observed, such as high pharate lethality
361 in *mld^{DTS-3/+}* animals shifted to an RT mid-metamorphosis, the early temperature-
362 insensitivity period of *mld^{DTS-3/+}* animals shifted from a PT to an RT, and the inability of
363 20E-feeding to rescue *mld^{DTS-3/+}* pupae to adulthood at an RT (Holden et al., 1985).
364 Additionally, the time scale over which induction of *ecd* or *mld* loss-of-function leads to a
365 loss or reduction in the ecdysteroid biosynthetic capacity of the PG is unclear. Dissected
366 brain-RG complexes from *ecd^l* animals pre-incubated at an RT for 24 hr secrete

367 approximately 50% the level of E compared to those pre-incubated for 0 hr (Henrich et
368 al., 1987), suggesting ecdysteroid secretion is not quickly abolished upon inactivation of
369 *ecd*. Also, while Garen *et al.* (1977) found *ecd^l* larvae shifted to an RT 20–24 hrs pre-
370 pupariation have negligible levels of ecdysteroids at a time when control larvae produce
371 the prepupal peak, a very similar experiment with a 24 hr pre-pupariation PT-to-RT shift
372 by Belinski-Deutsch *et al.* (1983) found no difference in the prepupal titre. To our
373 knowledge, no published information exists on ecdysteroid titres in *mld^{DTS-3}* pharate
374 adults.

375

376 Regardless of these uncertainties around the *ecd^l* and *mld^{DTS-3}* alleles, given the lack of
377 ecdysone 20-monooxygenase activity after pupation discussed above, we argue our
378 interpretation of the data cited strongly point towards ecdysteroid biosynthesis not being
379 the proximal source of the ecdysteroids in the PAP.

380

381 **3.3. The recycling hypothesis**

382 Our third hypothesis for the source of the PAP is that it is derived from recycled
383 ecdysteroids: 20E synthesised earlier in development (from one or more of the
384 embryonic, larval, prepupal or pupal peaks; Fig. 1B) is inactivated, stored, reactivated,
385 and then released from a secondary source after pupation. This hypothesis—previously
386 proposed by Schwartz *et al.* (1984) and Dai & Gilbert (1991)—is consistent with the
387 data cited above, as it does not rely on the presence of active Shd enzyme during the
388 pupal–pharate adult transition; likewise, it does not require E biosynthesis in the PG or

389 any other alternative primary source. There is precedent for ecdysteroid recycling in
390 *Drosophila* and other insects: *D. melanogaster* embryos might contain maternal
391 ecdysteroid-acyl conjugates (see Section 2); eggs from multiple species of Lepidoptera
392 and Orthoptera contain maternal ecdysteroid-phosphate conjugates that are hydrolysed
393 during embryogenesis (reviewed in Rees, 1995; Sonobe and Ito, 2009); male
394 *Anopheles gambiae* mosquitoes gift an ecdysteroid-phosphate conjugate to females
395 that is then deconjugated to produce an active hormone that controls female mating
396 behaviour (Peng et al., 2022); and in the dipteran *Sarcophaga peregrina*, conjugates of
397 20E are produced by larvae and then hydrolysed during pupal development
398 (Moribayashi et al., 1985; Moribayashi and Ohtaki, 1980, 1978), although it is unclear if
399 this is the only source of 20E during this period.

400

401 Published experimental data suggest a secondary source of the *Drosophila* PAP is
402 highly plausible, but arguably stop short of supporting it conclusively. The high ratio of E
403 to 20E found after pupation (An et al., 2017; Handler, 1982; Lavrynenko et al., 2015; but
404 see Bainbridge and Bownes, 1988 for conflicting data) in the absence of Shd activity
405 (Mitchell and Smith, 1988) is consistent with 20E being produced by the reactivation of
406 inactive ecdysteroids (such as conjugates or other metabolites) while the degenerating
407 PG produces a modest but significant amount of E that fails to be 20-hydroxylated.
408 Additionally, according to Pak & Gilbert (1987; but see Lavrynenko et al., 2015 for
409 conflicting data, which may be due to ecdysteroid extraction and detection differences
410 between studies), 70% of the ecdysteroid content of white prepupae is comprised of

411 high- and low-polarity conjugates, the former of which at least can be hydrolysed *in vitro*
412 to predominantly 20-hydroxyecdysone and makisterone A, raising the possibility these
413 are being stored for use later in metamorphosis.

414

415 Another line of evidence that suggests PAP 20E might come from recycling is the ability
416 of exogenous 20E (supplemented in dietary media) to rescue a high proportion of *dib*
417 and *phm* Halloween null mutants to adulthood (Huynh et al., 2018). As ecdysteroid
418 secretion in discrete pulses is important for developmental transitions (Akagi et al.,
419 2016; Rewitz et al., 2010), reliance on exogenous 20E in biosynthetic mutants means
420 internal metabolic mechanisms can likely control the ecdysteroid titre independently of
421 intake. This is because: (1) it seems unlikely that larvae would produce discrete 20E
422 pulses by modulating their dietary intake of supplemented media, as they are
423 continuous feeders (Sewell et al., 1974) and rely on consistent nutrient intake to rapidly
424 grow to meet developmental checkpoints (reviewed in Koyama et al., 2013; Mirth et al.,
425 2020); and (2) while active ecdysteroid titres can be reduced by catabolism and
426 excretion (Guittard et al., 2011; Rewitz et al., 2010), increasing titres in post-feeding
427 stages (ie. prepupae, pupae and pharate adults) in the absence of biosynthesis likely
428 relies on recycling, as suggested by Schwartz *et al.* (1984). If such a mechanism exists
429 in chemically rescued biosynthetic mutants, it likely also exists in wild-type animals
430 during developmental stages where 20E biosynthesis is not active, such as—
431 hypothetically—in late pupae/pharate adults. This is independently supported by the
432 observation that feeding 20E to wild-type larvae increases 20E pulse titres during both

433 feeding and post-feeding stages—including the PAP—compared to non-20E-fed larvae
434 (Schwartz et al., 1984), which strongly suggests at least some of the PAP titre is derived
435 from 20E circulating during feeding stages.

436

437 As mentioned above, catabolism plays an important role in decreasing ecdysteroid
438 levels to produce pulse-like titres (Akagi et al., 2016; Rewitz et al., 2010). It could be
439 argued, however, that catabolism should also be considered in the context of increasing
440 hormone levels: a complete lack of ecdysteroid catabolism might allow sufficient 20E to
441 accumulate to produce a pulse, even during periods of very low ecdysteroid
442 biosynthesis and/or conversion, especially when ecdysteroid excretion is impossible,
443 such as the closed system of metamorphosis. If this is the case during the PAP, it might
444 preclude the explanatory need for a secondary source.

445

446 Enzymatically characterised ecdysteroid catabolic pathways in *Drosophila* are
447 effectively limited to 26-hydroxylation/carboxylation through Cyp18a1 (Guittard et al.,
448 2011; Rewitz et al., 2010) and 3-epimerisation via 3-oxidation through ecdysone
449 oxidase/Eo (Takeuchi et al., 2005). *Eo* mRNA appears absent at the start of the PAP
450 (Graveley et al., 2011), consistent with an absence of 3-epi-ecdysteroids during the
451 PAP (Lavrynenko et al., 2015), suggesting the catabolic pathway is not active. However,
452 *Cyp18a1* is either lowly or moderately transcribed at the start of the PAP (Bassett et al.,
453 1997; Graveley et al., 2011) and ecdysonic acids start accumulating before the pulse's
454 peak (Lavrynenko et al., 2015), suggesting some ecdysonic acid formation is likely to

455 be occurring during the start of the PAP and ecdysteroid catabolism is not completely
456 absent as the PAP titre accumulates. While a fuller understanding of ecdysteroid
457 catabolism during *Drosophila* metamorphosis, including assays of enzymatic activity,
458 would provide a more definitive answer to this argument, we contend that a slow
459 accumulation of biosynthesised and converted 20E through a lack of catabolism is
460 unlikely to be the source of the PAP.

461

462 Overall, our interpretation of all the data discussed above is that a recycling origin for
463 the PAP is the most plausible of the three hypotheses presented. However, there is a
464 clear need for further experimentation by the *Drosophila* endocrinology community to
465 formally test this hypothesis, which we discuss below.

466

467 **4. Experimentally testing the recycling hypothesis**

468 A straightforward step towards exploring the source of the PAP would be to directly test
469 the biosynthesis hypotheses by using modern, temporally specific gene or protein
470 inactivation techniques to disrupt one or more Halloween genes between the pupal
471 ecdysteroid pulse and the PAP, then quantifying the PAP 20E titre and subsequent
472 developmental progression. If these data reject a biosynthetic source of the PAP,
473 researchers should focus on exploring the possibility of a secondary source for PAP
474 20E more directly.

475

476 Where should researchers start looking for the components of an ecdysteroid recycling
477 system in *Drosophila*? The hypothetical PAP secondary source must be one of the
478 larval tissues retained through to the pupal–pharate adult transition, which includes the
479 Malpighian tubules (Gautam and Tapadia, 2010), dissociated larval fat body cells
480 (Aguila et al., 2007; Nelliott et al., 2006), larval oenocytes (Gutierrez et al., 2007),
481 histoblasts (Madhavan and Madhavan, 1980), non-PG cells of the RG (Dai and Gilbert,
482 1991), and the imaginal discs (albeit everted into adult structures by the time of the
483 PAP). Oenocytes are perhaps the most intriguing candidate as they secrete and/or
484 synthesise ecdysteroids in other insects (Ma and Philogène, 1985; Romer et al., 1974;
485 Romer and Bressel, 1994; Studinger and Willig, 1975) and have links to ecdysteroid
486 metabolism in *Drosophila* (Chiang et al., 2016; Huang et al., 2019).

487

488 The secondary source tissue must also express one or more ecdysteroid metabolic
489 enzymes throughout larval development and early metamorphosis and switch to the
490 expression of corresponding reactivating enzymes just before the pupal–pharate adult
491 molt. Biochemical data on ecdysteroid conjugation in *Drosophila* is limited, with
492 evidence only for phosphorylation, fatty acylation and acetylation at present. 26-
493 phosphorylation of 20,26-dihydroxy-E occurs constitutively in the S2 cell line (Guittard et
494 al., 2011); E 22-phosphate is present in adult ovaries (Grau et al., 1995; Pis et al.,
495 1995); putative 3-epi-20E 3-phosphate can be formed in 3rd instar larvae (Sommé-
496 Martin et al., 1988a); and putative 3-dehydro-E 2-phosphate can be formed by 3rd instar
497 larval homogenate (Hilton, 2004). Adult flies, but particularly females, produce 22-fatty

498 acyl conjugates of E in various tissues (Grau and Lafont, 1994a, 1994b), while 20E 22-
499 acetate can also be formed by adult female flies (Maróy et al., 1988). High- and low-
500 polarity conjugates that possibly correspond to some or all of these specific compounds
501 have also been identified in prepupae, adults and cell lines (Dübendorfer, 1986;
502 Dübendorfer and Maróy, 1986; Grau and Lafont, 1994a, 1994b; Pak and Gilbert, 1987).

503

504 Specific ecdysteroid conjugation enzymes have yet to be identified in *D. melanogaster*,
505 although the gene families responsible for phosphorylation, fatty acylation and
506 glycosylation in other species are all present in its genome (Ahn and Marygold, 2021;
507 Duan et al., 2020; Scanlan et al., 2020). While the ecdysteroid modification enzymes
508 Cyp18a1 (a 26-hydroxylase/carboxylase) and Ecdysone oxidase (which produces 3-
509 dehydroecdysteroids) have been characterised in *Drosophila* (Guittard et al., 2011;
510 Rewitz et al., 2010; Takeuchi et al., 2005), they are unlikely to be the main contributors
511 to a recycling system, as 26-carboxylated ecdysteroids are thought to be irreversible,
512 terminal catabolites (Lafont et al., 1983) and 3-dehydroecdysteroids might be functional
513 hormones (Baker et al., 2003; Sommé-Martin et al., 1990).

514

515 Membrane transporter proteins might also be important for the movement of active
516 ecdysteroids and/or their conjugates in a recycling system. Ecdysone Importer (Ecl) is
517 required for 20E import in *Drosophila* (Okamoto et al., 2018; Okamoto and Yamanaka,
518 2020) and would likely be highly expressed during the uptake stage of a secondary
519 source. Ecdysteroid secretion likely also requires active transport—in the PG, the

520 transporter Atet fills vesicles with E for exocytosis (Yamanaka et al., 2015), and the
521 20E-inducible transporter E23 has been proposed to be a 20E exporter (Hock et al.,
522 2000); either gene could be involved in secreting deconjugated 20E from a secondary
523 source.

524

525 Ultimately, to fully test the recycling hypothesis, researchers would need to: (1) conduct
526 *ex vivo* ecdysteroid secretion experiments to find the secondary source tissue during
527 the pupal–pharate adult transition; (2) determine the storage form(s) of 20E present in
528 this tissue; (3) discover the identities of the conjugation and deconjugation enzymes
529 using biochemical and reverse genetic experiments; and (4) determine the
530 developmental consequences of disrupting the ecdysteroid recycling system using
531 genetic methods.

532

533 **5. The importance of the PAP in other dipterans and insects**

534 Distinct pulses of ecdysteroids at the onset of pharate adult development—at least
535 some of which are known to be coincident with pupal–adult apolysis and are therefore
536 likely homologous to the *Drosophila* PAP—are present in species from at least seven
537 dipteran superfamilies, across both suborders (Table 1). Biochemical data from non-
538 *Drosophila* dipterans suggest a secondary source of the PAP may be present in some,
539 but not all, taxa. Ecdysteroid recycling during metamorphosis likely occurs in the flesh
540 fly *Sarcophaga peregrina*, in which 20E can be converted during late larval or early

541 pupal stages to an unidentified polar conjugate (or group of conjugates) called OA
542 (Moribayashi and Ohtaki, 1980, 1978). The OA titre increases until the time of the
543 presumed PAP in this species, after which it decreases as the 20E titre increases
544 (Moribayashi and Ohtaki, 1980). As purified OA can be converted to 20E *in vitro* and *in*
545 *vivo*, this strongly suggests OA is a storage conjugate of 20E formed in larvae that is
546 recycled during the PAP (Moribayashi et al., 1985; Moribayashi and Ohtaki, 1980), but it
547 is unknown whether this recycling is the only source of 20E during the PAP in *S.*
548 *peregrina*. In the blowfly *Protophormia terraenovae*, early pupae contain esterase-
549 sensitive apolar conjugates, which decrease in titre at the time of the PAP before
550 slightly increasing prior to adult eclosion (Gaudry et al., 2006); however, it is unclear if
551 hydrolysis of these conjugates *in vivo* can explain the free ecdysteroid titre of the PAP
552 nor if recycling is occurring during metamorphosis.

553

554 Experiments by Jenkins et al. (1992) strongly suggest the PG cells do not secrete
555 ecdysteroids in 4th instar larvae and pupae of the mosquito *Aedes aegypti*, and that a
556 non-PG biosynthetic source (or sources) present in both the thorax and abdomen is
557 active during this time, with no hydrolysable ecdysteroid conjugates detectable in these
558 tissues. These data appear to rule out a secondary source for the PAP in *A. aegypti* and
559 suggest the source of the PAP is not perfectly conserved in dipterans. This imperfect
560 conservation extends to other holometabolous insects, as PAP-like pulses are likely
561 also derived from biosynthesis in multiple lepidopteran species (Beydon et al., 1981;
562 Bodnaryk, 1985; Rewitz et al., 2006b; Sakurai et al., 1991).

563

564 Pupal diapause is common in dipterans and is found variably across many families,
565 including Sarcophagidae (Cruz et al., 1996; Denlinger, 1974; Denlinger et al., 1988;
566 Gibbs, 1975; Ohtaki and Takahashi, 1972; Zdárek and Denlinger, 1975), Oestridae
567 (Baird, 1972), Tachnidae (Wylie, 1977), Tephritidae (Neven and Yee, 2017;
568 Papanastasiou and Papadopoulos, 2014; Ragland et al., 2009; Wang et al., 2017;
569 Yasuda et al., 1994), Anthomyiidae (Hao et al., 2012; Johnsen and Gutierrez, 1997),
570 Muscidae (Lysyk and Moon, 2001), Hippoboscidae (Kaunisto et al., 2015), and
571 Drosophilidae (Goto et al., 1999; although not in *D. melanogaster*). A negative
572 association between the PAP and pupal diapause in dipterans has been pointed out
573 previously (Fraenkel and Hsiao, 1968; Ohtaki and Takahashi, 1972; Walker and
574 Denlinger, 1980; see also Table 1), as development ceases prior to pupal–adult
575 apolysis (Dowle et al., 2020; Papanastasiou and Papadopoulos, 2014; Richard et al.,
576 1987), the PAP is not produced in diapausing individuals (Moribayashi et al., 1988;
577 Ohtaki and Takahashi, 1972; Richard et al., 1987; Walker and Denlinger, 1980), and
578 injections of 20E are sufficient to terminate diapause (Baird, 1972; Chen et al., 2016;
579 Gibbs, 1976; Ždárek and Denlinger, 1987; Zdárek and Denlinger, 1975). Understanding
580 the mechanisms by which the PAP is produced in *Drosophila* will likely shed light on
581 pupal diapause initiation, maintenance and termination in other dipterans.

582

583 As the PAP controls many aspects of adult tissue development, understanding how this
584 pulse of ecdysteroids is produced and regulated is important for the use of *D.*

585 *melanogaster* as an endocrinological model for other insects, especially as the
586 molecular pathways that control the secretion of E from the PG (reviewed in
587 Kannangara et al., 2021; Pan et al., 2021) and a hypothetical recycling system during
588 metamorphosis could substantially differ. Differences in the use of a PAP secondary
589 source between insects, as highlighted above, could also present novel, taxon-specific
590 pest control opportunities.

591

592 It is also tempting to speculate that regulation of the PAP could be a method through
593 which *Drosophila* spp. and related dipterans adaptively specify the adult phenotype in
594 relation to the juvenile environment. The timing of the PAP acts as a 'switch' for
595 seasonal polyphenism in multiple butterfly species (Brakefield et al., 1998; Mateus et
596 al., 2014; Oostra et al., 2014, 2011; Rountree and Nijhout, 1995); a similar mechanism
597 could exist in the pest *Drosophila suzukii*, which develops into winter and summer adult
598 morphs in response to environmental conditions (Shearer et al., 2016), and in other
599 dipterans that also exhibit seasonal polyphenism (Mielczarek et al., 2016).

600

601 **6. Conclusion**

602 In summary, while the source of 20E during the PAP is an unresolved question in
603 *Drosophila* biology, we contend that multiple lines of existing data, synthesised here for
604 the first time, support the hypothesis of a secondary source for this ecdysteroid pulse
605 over a traditional primary source such as the PG. This 20E recycling hypothesis, first

606 proposed in the 1980s and virtually neglected in recent years, deserves dedicated
607 experimental testing, and the knowledge gained from such efforts will greatly enrich our
608 current understanding of insect endocrinology.

609 References

- 610 Aguilá, J.R., Suszko, J., Gibbs, A.G., Hoshizaki, D.K., 2007. The role of larval fat cells in adult *Drosophila*
611 *melanogaster*. *J. Exp. Biol.* 210, 956–963. <https://doi.org/10.1242/jeb.001586>
- 612 Ahn, S.-J., Marygold, S.J., 2021. The UDP-Glycosyltransferase Family in *Drosophila melanogaster*:
613 Nomenclature Update, Gene Expression and Phylogenetic Analysis. *Front. Physiol.* 12, 648481.
614 <https://doi.org/10.3389/fphys.2021.648481> PMID - 33815151
- 615 Akagi, K., Sarhan, M., Sultan, A.-R.S., Nishida, H., Koie, A., Nakayama, T., Ueda, H., 2016. A biological
616 timer in the fat body comprising Blimp-1, β Ftz-f1 and Shade regulates pupation timing in
617 *Drosophila melanogaster*. *Development* 143, 2410–2416. <https://doi.org/10.1242/dev.133595>
- 618 Ameku, T., Niwa, R., 2016. Mating-Induced Increase in Germline Stem Cells via the Neuroendocrine
619 System in Female *Drosophila*. *PLoS Genet.* 12, e1006123.
620 <https://doi.org/10.1371/journal.pgen.1006123>
- 621 Ameku, T., Yoshinari, Y., Fukuda, R., Niwa, R., 2017. Ovarian ecdysteroid biosynthesis and female
622 germline stem cells. *Fly* 127, 1–9. <https://doi.org/10.1080/19336934.2017.1291472>
- 623 An, P.N.T., Yamaguchi, M., Fukusaki, E., 2017. Metabolic profiling of *Drosophila melanogaster*
624 metamorphosis: a new insight into the central metabolic pathways. *Metabolomics* 13, e99519.
625 <https://doi.org/10.1007/s11306-017-1167-1>
- 626 Bainbridge, S.P., Bownes, M., 1988. Ecdysteroid titers during *Drosophila* metamorphosis. *Insect*
627 *Biochem.* 18, 185–197. [https://doi.org/10.1016/0020-1790\(88\)90023-6](https://doi.org/10.1016/0020-1790(88)90023-6)
- 628 Bainbridge, S.P., Bownes, M., 1981. Staging the metamorphosis of *Drosophila melanogaster*. *J. Embryol.*
629 *Exp. Morphol.* 66, 57–80.
- 630 Baird, C.R., 1972. Termination of pupal diapause in *Cuterebra tenebrosa* (Diptera: Cuterebridae) with
631 injections of ecdysterone. *J. Med. Entomol.* 9, 77–80. <https://doi.org/10.1093/jmedent/9.1.77>
- 632 Baker, K.D., Shewchuk, L.M., Kozlova, T., Makishima, M., Hassell, A., Wisely, B., Caravella, J.A.,
633 Lambert, M.H., Reinking, J.L., Krause, H., Thummel, C.S., Willson, T.M., Mangelsdorf, D.J.,
634 2003. The *Drosophila* Orphan Nuclear Receptor DHR38 Mediates an Atypical Ecdysteroid
635 Signaling Pathway. *Cell* 113, 731–742. [https://doi.org/10.1016/s0092-8674\(03\)00420-3](https://doi.org/10.1016/s0092-8674(03)00420-3)
- 636 Bassett, M.H., McCarthy, J.L., Waterman, M.R., Sliter, T.J., 1997. Sequence and developmental
637 expression of Cyp18, a member of a new cytochrome P450 family from *Drosophila*. *Mol. Cell.*
638 *Endocrinol.* 131, 39–49. [https://doi.org/10.1016/s0303-7207\(97\)00093-2](https://doi.org/10.1016/s0303-7207(97)00093-2)
- 639 Belinski-Deutsch, S., Busson, D., Lamour-Audit, C., Porcheron, P., Moriniere, M., Berreur, P., 1983.
640 Relations between ecdysteroid levels and pupal development in the *ecd-1* temperature-sensitive
641 mutant of *Drosophila melanogaster*. *J. Insect Physiol.* 29, 509–514. [https://doi.org/10.1016/0022-1910\(83\)90081-1](https://doi.org/10.1016/0022-1910(83)90081-1)
- 643 Berreur, P., Porcheron, P., Berreur-Bonnenfant, J., Simpson, P., 1979. Ecdysteroid levels and pupariation
644 in *drosophila melanogaster*. *J. Exp. Zool.* 210, 347–352. <https://doi.org/10.1002/jez.1402100218>
- 645 Beydon, P., Claret, J., Porcheron, P., Lafont, R., 1981. Biosynthesis and inactivation of ecdysone during
646 the pupal-adult development of the cabbage butterfly, *Pieris brassicae* L. *Steroids* 38, 633–650.
647 [https://doi.org/10.1016/0039-128x\(81\)90083-0](https://doi.org/10.1016/0039-128x(81)90083-0)
- 648 Blais, C., Blasco, T., Maria, A., Dauphin-Villemant, C., Lafont, R., 2010. Characterization of ecdysteroids
649 in *Drosophila melanogaster* by enzyme immunoassay and nano-liquid chromatography-tandem
650 mass spectrometry. *Journal of Chromatography B* 878, 925–932.
651 <https://doi.org/10.1016/j.jchromb.2010.02.018>

- 652 Bodnaryk, R.P., 1985. Ecdysteroid levels during post-diapause development and 20-hydroxyecdysone-
653 induced development in male pupae of *Mamestra configurata* Wlk. *J. Insect Physiol.* 31, 53–58.
654 [https://doi.org/10.1016/0022-1910\(85\)90042-3](https://doi.org/10.1016/0022-1910(85)90042-3)
- 655 Bownes, M., 1992. Why is there sequence similarity between insect yolk proteins and vertebrate lipases?
656 *J. Lipid Res.* 33, 777–790.
- 657 Bownes, M., 1989. The roles of juvenile hormone, ecdysone and the ovary in the control of *Drosophila*
658 vitellogenesis. *J. Insect Physiol.* 35, 409–413. [https://doi.org/10.1016/0022-1910\(89\)90115-7](https://doi.org/10.1016/0022-1910(89)90115-7)
- 659 Bownes, M., Dübendorfer, A., Smith, T., 1984. Ecdysteroids in adult males and females of *Drosophila*
660 *melanogaster*. *J. Insect Physiol.* 30, 823–830. [https://doi.org/10.1016/0022-1910\(84\)90019-2](https://doi.org/10.1016/0022-1910(84)90019-2)
- 661 Bownes, M., Shirras, A., Blair, M., Collins, J., Coulson, A., 1988. Evidence that insect embryogenesis is
662 regulated by ecdysteroids released from yolk proteins. *Proc. Natl. Acad. Sci. U. S. A.* 85, 1554–
663 1557. <https://doi.org/10.2307/31268>
- 664 Brakefield, P.M., Kesbeke, F., Koch, P.B., 1998. The regulation of phenotypic plasticity of eyespots in the
665 butterfly *Bicyclus anynana*. *Am. Nat.* 152, 853–860. <https://doi.org/10.1086/286213>
- 666 Brandão, A.D.S., do Amaral, J.B., Rezende-Teixeira, P., Hartfelder, K., Siviero, F., Machado-Santelli,
667 G.M., 2014. Cell death and tissue reorganization in *Rhynchosciara americana* (Sciaridae:
668 Diptera) metamorphosis and their relation to molting hormone titers. *Arthropod Struct. Dev.* 43,
669 511–522. <https://doi.org/10.1016/j.asd.2014.05.001>
- 670 Briers, T., Loof, A.D.E., 1980. The molting hormone activity in *Sarcophaga bullata* in relation to
671 metamorphosis and reproduction. *International Journal of Invertebrate Reproduction* 2, 363–372.
672 <https://doi.org/10.1080/01651269.1980.10553370>
- 673 Briers, T., van Beek, E., De Loof, A., 1983. Ecdysteroid activity during metamorphosis and in male and
674 female adults of four blowfly species. *Comparative Biochemistry and Physiology -- Part A:*
675 *Physiology* 74, 521–524. [https://doi.org/10.1016/0300-9629\(83\)90541-8](https://doi.org/10.1016/0300-9629(83)90541-8)
- 676 Buhler, K., Clements, J., Winant, M., Bolckmans, L., Vulsteke, V., Callaerts, P., 2018. Growth control
677 through regulation of insulin signalling by nutrition-activated steroid hormone in *Drosophila*.
678 *Development* 145. <https://doi.org/10.1242/dev.165654>
- 679 Buszczak, M., Freeman, M.R., Carlson, J.R., Bender, M., Cooley, L., Segraves, W.A., 1999. Ecdysone
680 response genes govern egg chamber development during mid-oogenesis in *Drosophila*.
681 *Development* 126, 4581–4589. [https://doi.org/10.1016/s0012-1606\(74\)80016-3](https://doi.org/10.1016/s0012-1606(74)80016-3)
- 682 Carvalho, M., Schwudke, D., Sampaio, J.L., Palm, W., Riezman, I., Dey, G., Gupta, G.D., Mayor, S.,
683 Riezman, H., Shevchenko, A., Kurzchalia, T.V., Eaton, S., 2010. Survival strategies of a sterol
684 auxotroph. *Development* 137, 3675–3685. <https://doi.org/10.1242/dev.044560>
- 685 Chanut-Delalande, H., Hashimoto, Y., Pelissier-Monier, A., Spokony, R., Dib, A., Kondo, T., Bohère, J.,
686 Niimi, K., Latapie, Y., Inagaki, S., Dubois, L., Valenti, P., Polesello, C., Kobayashi, S., Moussian,
687 B., White, K.P., Plaza, S., Kageyama, Y., Payre, F., 2014. Pri peptides are mediators of ecdysone
688 for the temporal control of development. *Nat. Cell Biol.* 127, 4115.
689 <https://doi.org/10.1038/ncb3052>
- 690 Chavez, V.M., Marques, G., Delbecq, J.P., Kobayashi, K., Hollingsworth, M., Burr, J., Natzle, J.E.,
691 O'Connor, M.B., 2000. The *Drosophila* disembodied gene controls late embryonic morphogenesis
692 and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels.
693 *Development* 127, 4115–4126.
- 694 Chen, Z., Dong, Y., Wang, Y., Andongma, A.A., Rashid, M.A., Krutmuang, P., Niu, C., 2016. Pupal
695 diapause termination in *Bactrocera minax*: an insight on 20-hydroxyecdysone induced phenotypic
696 and genotypic expressions. *Sci. Rep.* 6, 27440. <https://doi.org/10.1038/srep27440>

- 697 Chiang, Y.N., Tan, K.J., Chung, H., Lavrynenko, O., Shevchenko, A., Yew, J.Y., 2016. Steroid Hormone
698 Signaling Is Essential for Pheromone Production and Oenocyte Survival. *PLoS Genet.* 12,
699 e1006126. <https://doi.org/10.1371/journal.pgen.1006126>
- 700 Christesen, D., Yang, Y.T., Somers, J., Robin, C., Sztal, T., Batterham, P., Perry, T., 2017. Transcriptome
701 Analysis of *Drosophila melanogaster* Third Instar Larval Ring Glands Points to Novel Functions
702 and Uncovers a Cytochrome p450 Required for Development. *G3* 7, 467–479.
703 <https://doi.org/10.1534/g3.116.037333>
- 704 Clark, A.J., Block, K., 1959. The absence of sterol synthesis in insects. *J. Biol. Chem.* 234, 2578–2582.
- 705 Claudius, A.-K., Romani, P., Lamkemeyer, T., Jindra, M., Uhlirova, M., 2014. Unexpected Role of the
706 Steroid-Deficiency Protein Ecdysoneless in Pre-mRNA Splicing. *PLoS Genet.* 10, e1004287.
707 <https://doi.org/10.1371/journal.pgen.1004287>
- 708 Cruz, M.D.S., Robles, M.C.V., Thomas, G., 1996. In Vivo Rearing and Development of *Wohlfahrtia*
709 *magnifica* (Diptera: Sarcophagidae). *J. Med. Entomol.* 33, 586–591.
710 <https://doi.org/10.1093/jmedent/33.4.586> PMID - 8699452
- 711 Cummings, M.R., King, R.C., 1969. The cytology of the vitellogenic stages of oogenesis in *Drosophila*
712 *melanogaster*. I. General staging characteristics. *J. Morphol.* 128, 427–441.
713 <https://doi.org/10.1002/jmor.1051280404>
- 714 Dai, J., Gilbert, L.I., 1991. Metamorphosis of the corpus allatum and degeneration of the prothoracic
715 glands during the larval-pupal-adult transformation of *Drosophila melanogaster*: A
716 cytophysiological analysis of the ring gland. *Dev. Biol.* 144, 309–326.
717 [https://doi.org/10.1016/0012-1606\(91\)90424-2](https://doi.org/10.1016/0012-1606(91)90424-2)
- 718 Danielsen, E.T., Moeller, M.E., Dorry, E., Komura-Kawa, T., Fujimoto, Y., Troelsen, J.T., Herder, R.,
719 O'Connor, M.B., Niwa, R., Rewitz, K.F., 2014. Transcriptional Control of Steroid Biosynthesis
720 Genes in the *Drosophila* Prothoracic Gland by Ventral Veins Lacking and Knirps. *PLoS Genet.*
721 10, e1004343. <https://doi.org/10.1371/journal.pgen.1004343>
- 722 D'Avino, P.P., Thummel, C.S., 1998. *crooked legs* encodes a family of zinc finger proteins required for leg
723 morphogenesis and ecdysone-regulated gene expression during *Drosophila* metamorphosis.
724 *Development* 125, 1733–1745.
- 725 Delbecq, J.-P., Weidner, K., Hoffmann, K.H., 1990. Alternative sites for ecdysteroid production in
726 insects. *Invertebr. Reprod. Dev.* 18, 29–42. <https://doi.org/10.1080/07924259.1990.9672126>
- 727 Denlinger, D.L., 1974. Diapause potential in tropical flesh flies. *Nature* 252, 223–224.
728 <https://doi.org/10.1038/252223a0>
- 729 Denlinger, D.L., Chen, C.-P., Tanaka, S., 1988. The impact of diapause on the evolution of other life
730 history traits in flesh flies. *Oecologia* 77, 350–356. <https://doi.org/10.1007/bf00378041>
- 731 Domanitskaya, E., Anllo, L., Schüpbach, T., 2014. Phantom, a cytochrome P450 enzyme essential for
732 ecdysone biosynthesis, plays a critical role in the control of border cell migration in *Drosophila*.
733 *Dev. Biol.* 386, 408–418. <https://doi.org/10.1016/j.ydbio.2013.12.013>
- 734 Dowle, E.J., Powell, T.H.Q., Doellman, M.M., Meyers, P.J., Calvert, M.B., Walden, K.K.O., Robertson,
735 H.M., Berlocher, S.H., Feder, J.L., Hahn, D.A., Ragland, G.J., 2020. Genome-wide variation and
736 transcriptional changes in diverse developmental processes underlie the rapid evolution of
737 seasonal adaptation. *Proc. Natl. Acad. Sci. U. S. A.* 117, 23960–23969.
738 <https://doi.org/10.1073/pnas.2002357117>
- 739 Duan, H., Yang, X., Bu, Z., Li, X., Zhang, Z., Sun, W., 2020. Identification and Characterization of Genes
740 Involved in Ecdysteroid Esterification Pathway Contributing to the High 20-Hydroxyecdysone
741 Resistance of *Helicoverpa armigera*. *Front. Physiol.* 11, 253.

- 742 <https://doi.org/10.3389/fphys.2020.00508>
- 743 Dübendorfer, A., 1986. Ecdysone C20-hydroxylation and conjugate formation by *Drosophila*
744 *melanogaster* cell lines. *Insect Biochem.* 16, 645–651. [https://doi.org/10.1016/0020-](https://doi.org/10.1016/0020-1790(86)90007-7)
745 [1790\(86\)90007-7](https://doi.org/10.1016/0020-1790(86)90007-7)
- 746 Dübendorfer, A., Maróy, P., 1986. Ecdysteroid conjugation by tissues of adult females of *Drosophila*
747 *melanogaster*. *Insect Biochem.* 16, 109–113. [https://doi.org/10.1016/0020-1790\(86\)90084-3](https://doi.org/10.1016/0020-1790(86)90084-3)
- 748 Enya, S., Ameku, T., Igarashi, F., Iga, M., Kataoka, H., Shinoda, T., Niwa, R., 2014. A Halloween gene
749 *noppera-bo* encodes a glutathione S-transferase essential for ecdysteroid biosynthesis via
750 regulating the behaviour of cholesterol in *Drosophila*. *Sci. Rep.* 4, 6586.
751 <https://doi.org/10.1038/srep06586>
- 752 Feldlaufer, M.F., Weirich, G.F., Imberski, R.B., Svoboda, J.A., 1995. Ecdysteroid production in *Drosophila*
753 *melanogaster* reared on defined diets. *Insect Biochem. Mol. Biol.* 25, 709–712.
754 [https://doi.org/10.1016/0965-1748\(95\)00009-k](https://doi.org/10.1016/0965-1748(95)00009-k)
- 755 Fournet, F., Sannier, C., Moriniere, M., Porcheron, P., Monteny, N., 1995. Effects of two insect growth
756 regulators on ecdysteroid production in *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* 32,
757 588–593. <https://doi.org/10.1093/jmedent/32.5.588>
- 758 Fraenkel, G., Hsiao, C., 1968. Morphological and endocrinological aspects of pupal diapause in a fleshfly,
759 *Sarcophaga agrostoma*. *J. Insect Physiol.* 14, 707–718. [https://doi.org/10.1016/0022-](https://doi.org/10.1016/0022-1910(68)90229-1)
760 [1910\(68\)90229-1](https://doi.org/10.1016/0022-1910(68)90229-1)
- 761 Fristrom, D., 1993. The metamorphic development of the adult epidermis, in: Bate, M., Martinez-Arias, A.
762 (Eds.), *The Development of Drosophila Melanogaster*. Cold Spring Harbor Laboratory Press, pp.
763 843–897.
- 764 Fristrom, D., Liebrich, W., 1986. The hormonal coordination of cuticulin deposition and morphogenesis in
765 *Drosophila* imaginal discs in vivo and in vitro. *Dev. Biol.* 114, 1–11. [https://doi.org/10.1016/0012-](https://doi.org/10.1016/0012-1606(86)90378-7)
766 [1606\(86\)90378-7](https://doi.org/10.1016/0012-1606(86)90378-7)
- 767 Fristrom, J.W., Logan, W.R., Murphy, C., 1973. The synthetic and minimal culture requirements for
768 evagination of imaginal discs of *Drosophila melanogaster* in vitro. *Dev. Biol.* 33, 441–456.
769 [https://doi.org/10.1016/0012-1606\(73\)90149-8](https://doi.org/10.1016/0012-1606(73)90149-8)
- 770 Fujinaga, D., Gu, J., Kawahara, H., Ogihara, M.H., Kojima, I., Takeshima, M., Kataoka, H., 2020. Twenty-
771 hydroxyecdysone produced by dephosphorylation and ecdysteroidogenesis regulates early
772 embryonic development in the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* 103491.
773 <https://doi.org/10.1016/j.ibmb.2020.103491>
- 774 Garen, A., Kauvar, L., Lepesant, J.-A., 1977. Roles of ecdysone in *Drosophila* development. *Proc. Natl.*
775 *Acad. Sci. U. S. A.* 74, 5099–5103. <https://doi.org/10.1073/pnas.74.11.5099>
- 776 Gaudry, E., Blais, C., Maria, A., Dauphin-Villemant, C., 2006. Study of steroidogenesis in pupae of the
777 forensically important blow fly *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera:
778 Calliphoridae). *Forensic Sci. Int.* 160, 27–34. <https://doi.org/10.1016/j.forsciint.2005.06.014>
- 779 Gautam, N.-K., Tapadia, M., 2010. Ecdysone signaling is required for proper organization and fluid
780 secretion of stellate cells in the Malpighian tubules of *Drosophila melanogaster*. *Int. J. Dev. Biol.*
781 54, 635–642. <https://doi.org/10.1387/ijdb.092910ng>
- 782 Gaziouva, I., Bonnette, P.C., Henrich, V.C., Jindra, M., 2004. Cell-autonomous roles of the ecdysoneless
783 gene in *Drosophila* development and oogenesis. *Development* 131, 2715–2725.
784 <https://doi.org/10.1242/dev.01143>
- 785 Gibbs, D., 1976. The initiation of adult development in *Sarcophaga argyrostoma* by β -ecdysone. *J. Insect*

- 786 Physiol. 22, 1195–1200. [https://doi.org/10.1016/0022-1910\(76\)90093-7](https://doi.org/10.1016/0022-1910(76)90093-7)
- 787 Gibbs, D., 1975. Reversal of pupal diapause in *Sarcophaga argyrostoma* by temperature shifts after
788 puparium formation. *J. Insect Physiol.* 21, 1179–1186. [https://doi.org/10.1016/0022-](https://doi.org/10.1016/0022-1910(75)90085-2)
789 1910(75)90085-2
- 790 Gilbert, L.I., Rewitz, K.F., 2009. The Function and Evolution of the Halloween Genes: The Pathway to the
791 Arthropod Molting Hormone, in: Smagghe, G. (Ed.), *Ecdysone: Structures and Functions*,
792 *Ecdysone: Structures and Functions*. Springer Netherlands, Dordrecht, pp. 231–269.
793 https://doi.org/10.1007/978-1-4020-9112-4_9
- 794 Goodwin, T.W., Horn, D.H.S., Karlson, P., Koolman, J., Nakanishi, K., Robbins, W.E., Siddall, J.B.,
795 Takemoto, T., 1978. Ecdysteroids: a new generic term. *Nature* 272, 122.
796 <https://doi.org/10.1038/272122a0>
- 797 Goto, S.G., Yoshida, T., Beppu, K., Kimura, M.T., 1999. Evolution of overwintering strategies in Eurasian
798 species of the *Drosophila obscura* species group. *Biol. J. Linn. Soc. Lond.* 68, 429–441.
799 <https://doi.org/10.1111/j.1095-8312.1999.tb01179.x>
- 800 Grau, V., Lafont, R., 1994a. The Distribution of Ecdysone Metabolites Within the Body of Adult
801 *Drosophila-Melanogaster* Females and Their Sites of Production. *J. Insect Physiol.* 40, 87–96.
802 [https://doi.org/10.1016/0022-1910\(94\)90115-5](https://doi.org/10.1016/0022-1910(94)90115-5)
- 803 Grau, V., Lafont, R., 1994b. Metabolism of ecdysone and 20-hydroxyecdysone in adult *drosophila*
804 *melanogaster*. *Insect Biochem. Mol. Biol.* 24, 49–58. [https://doi.org/10.1016/0965-](https://doi.org/10.1016/0965-1748(94)90122-8)
805 1748(94)90122-8
- 806 Grau, V., Pis, J., Lafont, R., Others, 1995. Ovary-specific interaction of ecdysone 22-phosphate with
807 proteins in adult *Drosophila melanogaster* (Diptera: Drosophilidae). *Eur. J. Entomol.* 92, 189–189.
- 808 Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren,
809 M.J., Boley, N., Booth, B.W., Brown, J.B., Cherbas, L., Davis, C.A., Dobin, A., Li, R., Lin, W.,
810 Malone, J.H., Mattiuzzo, N.R., Miller, D., Sturgill, D., Tuch, B.B., Zaleski, C., Zhang, D.,
811 Blanchette, M., Dudoit, S., Eads, B., Green, R.E., Hammonds, A., Jiang, L., Kapranov, P.,
812 Langton, L., Perrimon, N., Sandler, J.E., Wan, K.H., Willingham, A., Zhang, Y., Zou, Y., Andrews,
813 J., Bickel, P.J., Brenner, S.E., Brent, M.R., Cherbas, P., Gingeras, T.R., Hoskins, R.A., Kaufman,
814 T.C., Oliver, B., Celniker, S.E., 2011. The developmental transcriptome of *Drosophila*
815 *melanogaster*. *Nature* 471, 473–479. <https://doi.org/10.1038/nature09715>
- 816 Guittard, E., Blais, C., Maria, A., Parvy, J.-P., Pasricha, S., Lumb, C., Lafont, R., Daborn, P.J., Dauphin-
817 Villemant, C., 2011. CYP18A1, a key enzyme of *Drosophila* steroid hormone inactivation, is
818 essential for metamorphosis. *Dev. Biol.* 349, 35–45. <https://doi.org/10.1016/j.ydbio.2010.09.023>
- 819 Gutierrez, E., Wiggins, D., Fielding, B., Gould, A.P., 2007. Specialized hepatocyte-like cells regulate
820 *Drosophila* lipid metabolism. *Nature* 445, 275–280. <https://doi.org/10.1038/nature05382>
- 821 Handler, A.M., 1982. Ecdysteroid titers during pupal and adult development in *Drosophila melanogaster*.
822 *Dev. Biol.* 93, 73–82. [https://doi.org/10.1016/0012-1606\(82\)90240-8](https://doi.org/10.1016/0012-1606(82)90240-8)
- 823 Hansson, L., Lambertsson, A., 1989. Steroid regulation of glue protein genes in *Drosophila melanogaster*.
824 *Hereditas* 110, 61–67. <https://doi.org/10.1111/j.1601-5223.1989.tb00418.x>
- 825 Hao, Y.-J., Li, W.-S., He, Z.-B., Si, F.-L., Ishikawa, Y., Chen, B., 2012. Differential gene expression
826 between summer and winter diapause pupae of the onion maggot *Delia antiqua*, detected by
827 suppressive subtractive hybridization. *J. Insect Physiol.* 58, 1444–1449.
828 <https://doi.org/10.1016/j.jinsphys.2012.08.010>
- 829 Henrich, V.C., Tucker, R.L., Maroni, G., Gilbert, L.I., 1987. The ecdysoneless (*ecd1ts*) mutation disrupts
830 ecdysteroid synthesis autonomously in the ring gland of *Drosophila melanogaster*. *Dev. Biol.* 120,

831 50–55. [https://doi.org/10.1016/0012-1606\(87\)90102-3](https://doi.org/10.1016/0012-1606(87)90102-3)

832 Hentze, J.L., Moeller, M.E., Jørgensen, A.F., Bengtsson, M.S., Bordoy, A.M., Warren, J.T., Gilbert, L.I.,
833 Andersen, O., Rewitz, K.F., 2013. Accessory Gland as a Site for Prothoracicotropic Hormone
834 Controlled Ecdysone Synthesis in Adult Male Insects. *PLoS One* 8, e55131.
835 <https://doi.org/10.1371/journal.pone.0055131.s002>

836 Hill, R.J., Billas, I.M.L., Bonneton, F., Graham, L.D., Lawrence, M.C., 2013. Ecdysone Receptors: From
837 the Ashburner Model to Structural Biology*. *Annu. Rev. Entomol.* 58, 251–271.
838 <https://doi.org/10.1146/annurev-ento-120811-153610>

839 Hilton, J.P., 2004. Isolation and characterization of ecdysteroid phosphotransferases. University of
840 Liverpool.

841 Hobson, R.P., 1935a. On a fat-soluble growth factor required by blow-fly larvae: Identity of the growth
842 factor with cholesterol. *Biochem. J.* 29, 2023–2026. <https://doi.org/10.1042/bj0292023>

843 Hobson, R.P., 1935b. On a fat-soluble growth factor required by blow-fly larvae: Distribution and
844 properties. *Biochem. J.* 29, 1292–1296. <https://doi.org/10.1042/bj0291292>

845 Hock, T., Cottrill, T., Keegan, J., Garza, D., 2000. The E23 early gene of *Drosophila* encodes an
846 ecdysone-inducible ATP-binding cassette transporter capable of repressing ecdysone-mediated
847 gene activation. *Proc. Natl. Acad. Sci. U. S. A.* 97, 9519–9524.
848 <https://doi.org/10.1073/pnas.160271797>

849 Holden, J.J., Suzuki, D.T., 1973. Temperature-sensitive mutations in *Drosophila melanogaster*. XII. The
850 genetic and developmental effects of dominant lethals on chromosome 3. *Genetics* 73, 445–458.
851 <https://doi.org/10.1093/genetics/73.3.445>

852 Holden, J.J.A., Walker, V.K., Maroy, P., Watson, K.L., White, B.N., Gausz, J., 1985. Analysis of molting
853 and metamorphosis in the ecdysteroid-deficient mutant L(3)3DTS of *Drosophila melanogaster*.
854 *Dev. Genet.* 6, 153–162. <https://doi.org/10.1002/dvg.1020060302>

855 Huang, K., Chen, W., Zhu, F., Li, P.W.-L., Kapahi, P., Bai, H., 2019. RiboTag translomic profiling of
856 *Drosophila* oenocytes under aging and induced oxidative stress. *BMC Genomics* 20, 50.
857 <https://doi.org/10.1186/s12864-018-5404-4>

858 Huynh, N., Zeng, J., Liu, W., King-Jones, K., 2018. A *Drosophila* CRISPR/Cas9 Toolkit for Conditionally
859 Manipulating Gene Expression in the Prothoracic Gland as a Test Case for Polytene Tissues. *G3: Genes/Genomes/Genetics* 8, 3593–3605. <https://doi.org/10.1534/g3.118.200539> PMID -
860 30213867
861

862 Ishimoto, H., Kitamoto, T., 2010. The Steroid Molting Hormone Ecdysone Regulates Sleep in Adult
863 *Drosophila melanogaster*. *Genetics* 185, 269–281. <https://doi.org/10.1534/genetics.110.114587>
864 PMID - 20215472

865 Ishimoto, H., Wang, Z., Rao, Y., Wu, C.-F., Kitamoto, T., 2012. A novel role for ecdysone in *Drosophila*
866 conditioned behavior: linking GPCR-mediated non-canonical steroid action to cAMP signaling in
867 the adult brain. *PLoS Genet.* 9, e1003843–e1003843.
868 <https://doi.org/10.1371/journal.pgen.1003843>

869 Jenkins, S.P., Brown, M.R., Lea, A.O., 1992. Inactive prothoracic glands in larvae and pupae of *Aedes*
870 *aegypti*: Ecdysteroid release by tissues in the thorax and abdomen. *Insect Biochem. Mol. Biol.* 22,
871 553–559. [https://doi.org/10.1016/0965-1748\(92\)90032-a](https://doi.org/10.1016/0965-1748(92)90032-a)

872 Johnsen, S., Gutierrez, A.P., 1997. Induction and Termination of Winter Diapause in a Californian Strain
873 of the Cabbage Maggot (Diptera: Anthomyiidae). *Environ. Entomol.* 26, 84–90.
874 <https://doi.org/10.1093/ee/26.1.84>

- 875 Kamiyama, T., Niwa, R., 2022. Transcriptional Regulators of Ecdysteroid Biosynthetic Enzymes and Their
876 Roles in Insect Development. *Front. Physiol.* 13. <https://doi.org/10.3389/fphys.2022.823418>
- 877 Kannangara, J.R., Mirth, C.K., Warr, C.G., 2021. Regulation of ecdysone production in *Drosophila* by
878 neuropeptides and peptide hormones. *Open Biol.* 11, 200373.
879 <https://doi.org/10.1098/rsob.200373>
- 880 Kaunisto, S., Härkönen, L., Rantala, M.J., Kortet, R., 2015. Early-life temperature modifies adult
881 encapsulation response in an invasive ectoparasite. *Parasitology* 142, 1290–1296.
882 <https://doi.org/10.1017/s0031182015000591>
- 883 King, R.C., Rubinson, A.C., Smith, R.F., 1956. Oogenesis in adult *Drosophila melanogaster*. *Growth* 20,
884 121–157.
- 885 Knapp, E., Sun, J., 2017. Steroid signaling in mature follicles is important for *Drosophila* ovulation. *Proc.*
886 *Natl. Acad. Sci. U. S. A.* 114, 699–704. <https://doi.org/10.1073/pnas.1614383114>
- 887 Koyama, T., Mendes, C.C., Mirth, C.K., 2013. Mechanisms regulating nutrition-dependent developmental
888 plasticity through organ-specific effects in insects. *Front. Physiol.* 4, 263.
889 <https://doi.org/10.3389/fphys.2013.00263>
- 890 Koyama, T., Rodrigues, M.A., Athanasiadis, A., Shingleton, A.W., Mirth, C.K., 2014. Nutritional control of
891 body size through FoxO-Ultraspiracle mediated ecdysone biosynthesis. *Elife* 3, 237.
892 <https://doi.org/10.7554/elife.03091>
- 893 Kozlova, T., Thummel, C.S., 2003. Essential roles for ecdysone signaling during *Drosophila* mid-
894 embryonic development. *Science* 301, 1911–1914. <https://doi.org/10.1126/science.1087419>
- 895 Lafont, R., Blais, C., Beydon, P., Modde, J.-F., Enderle, U., Koolman, J., 1983. Conversion of ecdysone
896 and 20-Hydroxyecdysone into 26-OIC derivatives is a major pathway in larvae and pupae of
897 species from three insect orders. *Arch. Insect Biochem. Physiol.* 1, 41–58.
898 <https://doi.org/10.1002/arch.940010106>
- 899 Lam, G., Hall, B.L., Bender, M., Thummel, C.S., 1999. DHR3 is required for the prepupal-pupal transition
900 and differentiation of adult structures during *Drosophila* metamorphosis. *Dev. Biol.* 212, 204–216.
901 <https://doi.org/10.1006/dbio.1999.9343>
- 902 Lavrynenko, O., Rodenfels, J., Carvalho, M., Dye, N.A., Lafont, R., Eaton, S., Shevchenko, A., 2015. The
903 ecdysteroidome of *Drosophila*: influence of diet and development. *Development* 142, 3758–3768.
904 <https://doi.org/10.1242/dev.124982>
- 905 Lawrence, P.O., Hagedorn, H.H., Wheelock, G., 1984. Ecdysteroid levels and integument changes in
906 post-embryonic stages of *Anastrepha suspensa*. *J. Insect Physiol.* 30, 713–719.
907 [https://doi.org/10.1016/0022-1910\(84\)90035-0](https://doi.org/10.1016/0022-1910(84)90035-0)
- 908 Leader, D.P., Krause, S.A., Pandit, A., Davies, S.A., Dow, J.A.T., 2018. FlyAtlas 2: a new version of the
909 *Drosophila melanogaster* expression atlas with RNA-Seq, miRNA-Seq and sex-specific data.
910 *Nucleic Acids Res.* 46, D809–D815. <https://doi.org/10.1093/nar/gkx976>
- 911 Lee, G., Park, J.H., 2021. Programmed cell death reshapes the central nervous system during
912 metamorphosis in insects. *Curr Opin Insect Sci* 43, 39–45.
913 <https://doi.org/10.1016/j.cois.2020.09.015>
- 914 Liu, S., Li, K., Gao, Y., Liu, X., Chen, W., Ge, W., Feng, Q., Palli, S.R., Li, S., 2017. Antagonistic actions
915 of juvenile hormone and 20-hydroxyecdysone within the ring gland determine developmental
916 transitions in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 80, 201716897.
917 <https://doi.org/10.1073/pnas.1716897115>
- 918 Lysyk, T.J., Moon, R.D., 2001. Diapause Recruitment and Survival of Overwintering *Haematobia irritans*

- 919 (Diptera: Muscidae). *Environ. Entomol.* 30, 1090–1097. [https://doi.org/10.1603/0046-225x-](https://doi.org/10.1603/0046-225x-30.6.1090)
920 30.6.1090
- 921 Ma, L., Philogène, B.J.R., 1985. Oenocyte and prothoracic gland activity in *Manduca sexta* under varying
922 photoperiod and light conditions. *Experientia* 41, 935–938. <https://doi.org/10.1007/bf01970021>
- 923 Madhavan, M.M., Madhavan, K., 1980. Morphogenesis of the epidermis of adult abdomen of *Drosophila*.
924 *J. Embryol. Exp. Morphol.* 60, 1–31.
- 925 Malita, A., Rewitz, K.F., 2021. Interorgan communication in the control of metamorphosis. *Curr Opin*
926 *Insect Sci* 43, 54–62. <https://doi.org/10.1016/j.cois.2020.10.005>
- 927 Margam, V.M., Gelman, D.B., Palli, S.R., 2006. Ecdysteroid titers and developmental expression of
928 ecdysteroid-regulated genes during metamorphosis of the yellow fever mosquito, *Aedes aegypti*
929 (Diptera: Culicidae). *J. Insect Physiol.* 52, 558–568. <https://doi.org/10.1016/j.jinsphys.2006.02.003>
- 930 Maróy, P., Kaufmann, G., Dübendorfer, A., 1988. Embryonic ecdysteroids of *Drosophila melanogaster*. *J.*
931 *Insect Physiol.* 34, 633–637. [https://doi.org/10.1016/0022-1910\(88\)90071-6](https://doi.org/10.1016/0022-1910(88)90071-6)
- 932 Martín-Vega, D., Simonsen, T.J., Hall, M.J.R., 2017. Looking into the puparium: Micro-CT visualization of
933 the internal morphological changes during metamorphosis of the blow fly, *Calliphora vicina*, with
934 the first quantitative analysis of organ development in cyclorrhaphous dipterans. *J. Morphol.* 278,
935 629–651. <https://doi.org/10.1002/jmor.20660>
- 936 Mateus, A.R.A., Marques-Pita, M., Oostra, V., Lafuente, E., Brakefield, P.M., Zwaan, B.J., Beldade, P.,
937 2014. Adaptive developmental plasticity: Compartmentalized responses to environmental cues
938 and to corresponding internal signals provide phenotypic flexibility. *BMC Biol.* 12, 1347.
939 <https://doi.org/10.1186/s12915-014-0097-x>
- 940 McBrayer, Z., Ono, H., Shimell, M., Parvy, J.-P., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-
941 Villemant, C., Gilbert, L.I., O'Connor, M.B., 2007. Prothoracicotropic hormone regulates
942 developmental timing and body size in *Drosophila*. *Dev. Cell* 13, 857–871.
943 <https://doi.org/10.1016/j.devcel.2007.11.003>
- 944 Mielczarek, L.E., Oleksa, A., Meyza, K., Tofilski, A., 2016. Seasonal polyphenism in *Eristalis pertinax*
945 (Diptera: Syrphidae). *Eur. J. Entomol.* 113, 489–496. <https://doi.org/10.14411/eje.2016.064>
- 946 Mirth, C., 2005. Ecdysteroid control of metamorphosis in the differentiating adult leg structures of
947 *Drosophila melanogaster*. *Dev. Biol.* 278, 163–174. <https://doi.org/10.1016/j.ydbio.2004.10.026>
- 948 Mirth, C.K., Saunders, T.E., Amourda, C., 2020. Growing Up in a Changing World: Environmental
949 Regulation of Development in Insects. *Annu. Rev. Entomol.* 66, 1–19.
950 <https://doi.org/10.1146/annurev-ento-041620-083838> PMID - 32822557
- 951 Mitchell, M.J., Smith, S.L., 1988. Ecdysone 20-monooxygenase activity throughout the life cycle of
952 *Drosophila melanogaster*. *Gen. Comp. Endocrinol.* 72, 467–470. [https://doi.org/10.1016/0016-](https://doi.org/10.1016/0016-6480(88)90170-0)
953 6480(88)90170-0
- 954 Moribayashi, A., Kurahashi, H., Ohtaki, T., 1988. Different profiles of ecdysone secretion and its
955 metabolism between diapause- and nondiapause-destined cultures of the fleshfly, *Boettcherisca*
956 *peregrina*. *Comp. Biochem. Physiol. A Comp. Physiol.* 91, 157–164. [https://doi.org/10.1016/0300-](https://doi.org/10.1016/0300-9629(88)91609-x)
957 9629(88)91609-x
- 958 Moribayashi, A., Kurahashi, H., Ohtaki, T., 1985. Comparative studies on ecdysone metabolism between
959 mature larvae and pharate pupae in the fleshfly, *Sarcophaga peregrina*. *Arch. Insect Biochem.*
960 *Physiol.* 2, 237–250. <https://doi.org/10.1002/arch.940020303>
- 961 Moribayashi, A., Ohtaki, T., 1980. Inactivation and reactivation of 20-hydroxyecdysone during pupal-adult
962 development of the flesh fly, *Sarcophaga peregrina*. *Jpn. J. Med. Sci. Biol.* 33, 189–201.

- 963 <https://doi.org/10.7883/yoken1952.33.189>
- 964 Moribayashi, A.F., Ohtaki, T., 1978. Inactivation of ecdysone and the possible feed back control of the
965 titre during pupation of *Sarcophaga peregrina*. *J. Insect Physiol.* 24, 279–284.
966 [https://doi.org/10.1016/0022-1910\(78\)90023-9](https://doi.org/10.1016/0022-1910(78)90023-9)
- 967 Namiki, T., Niwa, R., Sakudoh, T., Shirai, K.-I., Takeuchi, H., Kataoka, H., 2005. Cytochrome P450
968 CYP307A1/Spook: A regulator for ecdysone synthesis in insects. *Biochem. Biophys. Res.*
969 *Commun.* 337, 367–374. <https://doi.org/10.1016/j.bbrc.2005.09.043>
- 970 Nelliot, A., Bond, N., Hoshizaki, D.K., 2006. Fat-body remodeling in *Drosophila melanogaster*. *Genesis*
971 44, 396–400. <https://doi.org/10.1002/dvg.20229>
- 972 Neubueser, D., Warren, J.T., Gilbert, L.I., Cohen, S.M., 2005. molting defective is required for ecdysone
973 biosynthesis. *Dev. Biol.* 280, 362–372. <https://doi.org/10.1016/j.ydbio.2005.01.023>
- 974 Neven, L.G., Yee, W.L., 2017. Impact of Prolonged Absence of Low Temperature on Adult Eclosion
975 Patterns of Western Cherry Fruit Fly (Diptera: Tephritidae). *Environ. Entomol.* 46, 708–713.
976 <https://doi.org/10.1093/ee/nvx064>
- 977 Niwa, R., Namiki, T., Ito, K., Shimada-Niwa, Y., Kiuchi, M., Kawaoka, S., Kayukawa, T., Banno, Y.,
978 Fujimoto, Y., Shigenobu, S., Kobayashi, S., Shimada, T., Katsuma, S., Shinoda, T., 2010. Non-
979 molting glossy/shroud encodes a short-chain dehydrogenase/reductase that functions in the
980 “Black Box” of the ecdysteroid biosynthesis pathway. *Development* 137, 1991–1999.
981 <https://doi.org/10.1242/dev.045641>
- 982 Niwa, R., Niwa, Y.S., 2014. Enzymes for ecdysteroid biosynthesis: their biological functions in insects and
983 beyond. *Biosci. Biotechnol. Biochem.* 78, 1283–1292.
984 <https://doi.org/10.1080/09168451.2014.942250>
- 985 Nunes, C., Sucena, É., Koyama, T., 2021. Endocrine regulation of immunity in insects. *FEBS J.* 288,
986 3928–3947. <https://doi.org/10.1111/febs.15581>
- 987 Ohtaki, T., Takahashi, M., 1972. Induction and termination of pupal diapause in relation to the change of
988 ecdysone titer in the fleshfly, *Sarcophaga peregrina*. *Jpn. J. Med. Sci. Biol.* 25, 369–376.
989 <https://doi.org/10.7883/yoken1952.25.369>
- 990 Okamoto, N., Viswanatha, R., Bittar, R., Li, Z., Haga-Yamanaka, S., Perrimon, N., Yamanaka, N., 2018. A
991 Membrane Transporter Is Required for Steroid Hormone Uptake in *Drosophila*. *Dev. Cell.*
992 <https://doi.org/10.1016/j.devcel.2018.09.012>
- 993 Okamoto, N., Yamanaka, N., 2020. Steroid Hormone Entry into the Brain Requires a Membrane
994 Transporter in *Drosophila*. *Curr. Biol.* 30, 359-366.e3. <https://doi.org/10.1016/j.cub.2019.11.085>
- 995 O'Neill, M.P., Holman, G.M., Wright, J.E., 1977. β -ecdysone levels in pharate pupae of the stable fly,
996 *Stomoxys calcitrans* and interaction with the chitin inhibitor diflubenzuron. *J. Insect Physiol.* 23,
997 1243–1244. [https://doi.org/10.1016/0022-1910\(77\)90065-8](https://doi.org/10.1016/0022-1910(77)90065-8)
- 998 Ono, H., 2014. Ecdysone differentially regulates metamorphic timing relative to 20-hydroxyecdysone by
999 antagonizing juvenile hormone in *Drosophila melanogaster*. *Dev. Biol.* 391, 32–42.
1000 <https://doi.org/10.1016/j.ydbio.2014.04.004>
- 1001 Ono, H., Rewitz, K.F., Shinoda, T., Itoyama, K., Petryk, A., Rybczynski, R., Jarcho, M., Warren, J.T.,
1002 Marqués, G., Shimell, M.J., Gilbert, L.I., O'Connor, M.B., 2006. Spook and Spookier code for
1003 stage-specific components of the ecdysone biosynthetic pathway in Diptera. *Dev. Biol.* 298, 555–
1004 570. <https://doi.org/10.1016/j.ydbio.2006.07.023>
- 1005 Oostra, V., Jong, M.A. de, Invergo, B.M., Kesbeke, F., Wende, F., Brakefield, P.M., Zwaan, B.J., 2011.
1006 Translating environmental gradients into discontinuous reaction norms via hormone signalling in a

- 1007 polyphenic butterfly. *Proceedings of the Royal Society of London B: Biological Sciences* 278,
1008 789–797. <https://doi.org/10.1098/rspb.2010.1560>
- 1009 Oostra, V., Mateus, A.R.A., Burg, K.R.L. van der, Piessens, T., van Eijk, M., Brakefield, P.M., Beldade, P.,
1010 Zwaan, B.J., 2014. Ecdysteroid Hormones Link the Juvenile Environment to Alternative Adult Life
1011 Histories in a Seasonal Insect. *Am. Nat.* 184, E79–E92. <https://doi.org/10.1086/677260>
- 1012 Ou, Q., Magico, A., King-Jones, K., 2011. Nuclear Receptor DHR4 Controls the Timing of Steroid
1013 Hormone Pulses During *Drosophila* Development. *PLoS Biol.* 9, e1001160.
1014 <https://doi.org/10.1371/journal.pbio.1001160>
- 1015 Pak, M.D., Gilbert, L.I., 1987. A Developmental Analysis of Ecdysteroids During the Metamorphosis of
1016 *Drosophila Melanogaster*. *J. Liq. Chromatogr.* 10, 2591–2611.
1017 <https://doi.org/10.1080/01483918708066815>
- 1018 Pan, X., Connacher, R.P., O'Connor, M.B., 2021. Control of the insect metamorphic transition by
1019 ecdysteroid production and secretion. *Curr Opin Insect Sci* 43, 11–20.
1020 <https://doi.org/10.1016/j.cois.2020.09.004>
- 1021 Papanastasiou, S.A., Papadopoulou, N.T., 2014. Description of *Rhagoletis cerasi* (Diptera: Tephritidae)
1022 pupal developmental stages: indications of prolonged diapause. *J. Insect Sci.* 14, 156.
1023 <https://doi.org/10.1093/jisesa/ieu018>
- 1024 Parvy, J.-P., Blais, C., Bernard, F., Warren, J.T., Petryk, A., Gilbert, L.I., O'Connor, M.B., Dauphin-
1025 Villemant, C., 2005. A role for betaFTZ-F1 in regulating ecdysteroid titers during post-embryonic
1026 development in *Drosophila melanogaster*. *Dev. Biol.* 282, 84–94.
1027 <https://doi.org/10.1016/j.ydbio.2005.02.028>
- 1028 Peng, D., Kakani, E.G., Mameli, E., Vidoudez, C., Mitchell, S.N., Merrihew, G.E., MacCoss, M.J., Adams,
1029 K., Rinvee, T.A., Shaw, W.R., Catteruccia, F., 2022. A male steroid controls female sexual
1030 behaviour in the malaria mosquito. *Nature* 1–5. <https://doi.org/10.1038/s41586-022-04908-6>
- 1031 Petruccelli, E., Lark, A., Mrkvicka, J.A., Kitamoto, T., 2020. Significance of DopEcR, a G-protein coupled
1032 dopamine/ecdysteroid receptor, in physiological and behavioral response to stressors. *J.*
1033 *Neurogenet.* 265, 1–14. <https://doi.org/10.1080/01677063.2019.1710144>
- 1034 Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.-P., Li, Y., Dauphin-
1035 Villemant, C., O'Connor, M.B., 2003. Shade is the *Drosophila* P450 enzyme that mediates the
1036 hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl.*
1037 *Acad. Sci. U. S. A.* 100, 13773–13778. <https://doi.org/10.1073/pnas.2336088100>
- 1038 Pis, J., Girault, J.P., Grau, V., Lafont, R., 1995. Analysis of ecdysteroid conjugates: Chromatographic
1039 characterization of sulfates, phosphates and glucosides. *EUROPEAN JOURNAL OF* 92, 41–41.
- 1040 Ragland, G.J., Fuller, J., Feder, J.L., Hahn, D.A., 2009. Biphasic metabolic rate trajectory of pupal
1041 diapause termination and post-diapause development in a tephritid fly. *J. Insect Physiol.* 55, 344–
1042 350. <https://doi.org/10.1016/j.jinsphys.2008.12.013>
- 1043 Redfern, C.P.F., 1983. Ecdysteroid synthesis by the ring gland of *Drosophila melanogaster* during late-
1044 larval, prepupal and pupal development. *J. Insect Physiol.* 29, 65–71.
1045 [https://doi.org/10.1016/0022-1910\(83\)90107-5](https://doi.org/10.1016/0022-1910(83)90107-5)
- 1046 Redfern, C.P.F., Bownes, M., 1983. Pleiotropic effects of the 'ecdysoneless-1' mutation of *Drosophila*
1047 *melanogaster*. *Mol. Gen. Genet.* 189, 432–440. <https://doi.org/10.1007/BF00325905>
- 1048 Rees, H.H., 1995. Ecdysteroid Biosynthesis and Inactivation in Relation to Function. *Eur. J. Entomol.* 92,
1049 9–39.
- 1050 Rewitz, K.F., Rybczynski, R., Warren, J.T., Gilbert, L.I., 2006a. The Halloween genes code for

- 1051 cytochrome P450 enzymes mediating synthesis of the insect moulting hormone. *Biochem. Soc.*
1052 *Trans.* 34, 1256–1260. <https://doi.org/10.1042/bst0341256>
- 1053 Rewitz, K.F., Rybczynski, R., Warren, J.T., Gilbert, L.I., 2006b. Developmental expression of *Manduca*
1054 *shade*, the P450 mediating the final step in molting hormone synthesis. *Mol. Cell. Endocrinol.*
1055 247, 166–174. <https://doi.org/10.1016/j.mce.2005.12.053>
- 1056 Rewitz, K.F., Yamanaka, N., Gilbert, L.I., O'Connor, M.B., 2009. The insect neuropeptide PTTH activates
1057 receptor tyrosine kinase torso to initiate metamorphosis. *Science* 326, 1403–1405.
1058 <https://doi.org/10.1126/science.1176450>
- 1059 Rewitz, K.F., Yamanaka, N., O'Connor, M.B., 2010. Steroid hormone inactivation is required during the
1060 juvenile-adult transition in *Drosophila*. *Dev. Cell* 19, 895–902.
1061 <https://doi.org/10.1016/j.devcel.2010.10.021>
- 1062 Rharrabe, K., Alla, S., Maria, A., Sayah, F., Lafont, R., 2007. Diversity of detoxification pathways of
1063 ingested ecdysteroids among phytophagous insects. *Arch. Insect Biochem. Physiol.* 65, 65–73.
1064 <https://doi.org/10.1002/arch.20191>
- 1065 Richard, D.S., Gilbert, L.I., 1991. Reversible juvenile hormone inhibition of ecdysteroid and juvenile
1066 hormone synthesis by the ring gland of *Drosophila melanogaster*. *Experientia* 47, 1063–1066.
1067 <https://doi.org/10.1007/BF01923343>
- 1068 Richard, D.S., Warrent, J.T., Saunders, D.S., Gilbert, L.I., 1987. Haemolymph ecdysteroid titres in
1069 diapause- and non-diapause-destined larvae and pupae of *Sarcophaga argyrostoma*. *Journal of*
1070 *Insect Physiology*. [https://doi.org/10.1016/0022-1910\(87\)90083-7](https://doi.org/10.1016/0022-1910(87)90083-7)
- 1071 Richards, G., 1981. The radioimmune assay of ecdysteroid titres in *drosophila melanogaster*. *Mol. Cell.*
1072 *Endocrinol.* 21, 181–197. [https://doi.org/10.1016/0303-7207\(81\)90013-7](https://doi.org/10.1016/0303-7207(81)90013-7)
- 1073 Riddiford, L.M., 1993. Hormones and *Drosophila* development, in: Bate, M., Martinez-Arias, A. (Eds.), *The*
1074 *Development of Drosophila Melanogaster*. Cold Spring Harbor Laboratory Press, pp. 899–939.
- 1075 Robinow, S., Talbot, W.S., Hogness, D.S., Truman, J.W., 1993. Programmed cell death in the *Drosophila*
1076 CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. *Development*
1077 119, 1251–1259. <https://doi.org/10.1242/dev.119.4.1251>
- 1078 Romer, F., Bressel, H.U., 1994. Secretion and Metabolism of Ecdysteroids by Oenocyte-Fat Body
1079 Complexes (Oefc) in Adult Males of *Gryllus-Bimaculatus* Deg (Insecta). *Zeitschrift fur*
1080 *Naturforschung C-Journal of Biosciences* 49, 871–880.
- 1081 Romer, F., Emmerich, H., Nowock, J., 1974. Biosynthesis of ecdysones in isolated prothoracic glands and
1082 oenocytes of *Tenebrio molitor* in vitro. *J. Insect Physiol.* 20, 1975–1987.
1083 [https://doi.org/10.1016/0022-1910\(74\)90105-x](https://doi.org/10.1016/0022-1910(74)90105-x)
- 1084 Rountree, D.B., Nijhout, H.F., 1995. Hormonal control of a seasonal polyphenism in *Precis coenia*
1085 (*Lepidoptera: Nymphalidae*). *J. Insect Physiol.* 41, 987–992. [https://doi.org/10.1016/0022-1910\(95\)00046-w](https://doi.org/10.1016/0022-1910(95)00046-w)
- 1087 Roy, S., Saha, T.T., Zou, Z., Raikhel, A.S., 2018. Regulatory Pathways Controlling Female Insect
1088 Reproduction. *Annu. Rev. Entomol.* 63, 489–511. <https://doi.org/10.1146/annurev-ento-020117-043258>
- 1090 Rubenstein, E.C., Kelly, T.J., Schwartz, M.B., Woods, C.W., 1982. In vitro synthesis and secretion of
1091 ecdysteroids by *Drosophila melanogaster* ovaries. *J. Exp. Zool. A Ecol. Genet. Physiol.* 223, 305–
1092 308. <https://doi.org/10.1002/jez.1402230313>
- 1093 Sakurai, S., Warren, J.T., Gilbert, L.I., 1991. Ecdysteroid synthesis and molting by the tobacco hornworm,
1094 *Manduca sexta*, in the absence of prothoracic glands. *Arch. Insect Biochem. Physiol.* 18, 13–36.

- 1095 <https://doi.org/10.1002/arch.940180103>
- 1096 Scanlan, J.L., Gledhill-Smith, R.S., Battlay, P., Robin, C., 2020. Genomic and transcriptomic analyses in
1097 *Drosophila* suggest that the ecdysteroid kinase-like (EcKL) gene family encodes the
1098 “detoxification-by-phosphorylation” enzymes of insects. *Insect Biochem. Mol. Biol.* 123, 103429.
1099 <https://doi.org/10.1016/j.ibmb.2020.103429>
- 1100 Schwartz, M.B., Imberski, R.B., Kelly, T.J., 1984. Analysis of Metamorphosis in *Drosophila-Melanogaster*
1101 - Characterization of Giant, an Ecdysteroid-Deficient Mutant. *Dev. Biol.* 103, 85–95.
1102 [https://doi.org/10.1016/0012-1606\(84\)90010-1](https://doi.org/10.1016/0012-1606(84)90010-1)
- 1103 Schwartz, M.B., Kelly, T.J., Imberski, R.B., Rubenstein, E.C., 1985. The effects of nutrition and
1104 methoprene treatment on ovarian ecdysteroid synthesis in *Drosophila melanogaster*. *J. Insect*
1105 *Physiol.* 31, 947–957. [https://doi.org/10.1016/0022-1910\(85\)90029-0](https://doi.org/10.1016/0022-1910(85)90029-0)
- 1106 Schwartz, M.B., Kelly, T.J., Woods, C.W., Imberski, R.B., 1989. Ecdysteroid fluctuations in adult
1107 *Drosophila melanogaster* caused by elimination of pupal reserves and synthesis by early
1108 vitellogenic ovarian follicles. *Insect Biochem.* 19, 243–249. [https://doi.org/10.1016/0020-1790\(89\)90068-1](https://doi.org/10.1016/0020-1790(89)90068-1)
- 1110 Schwedes, C.C., Carney, G.E., 2012. Ecdysone signaling in adult *Drosophila melanogaster*. *J. Insect*
1111 *Physiol.* 58, 293–302. <https://doi.org/10.1016/j.jinsphys.2012.01.013>
- 1112 Sewell, D., Burnet, B., Connolly, K., 1974. Genetic analysis of larval feeding behaviour in *Drosophila*
1113 *melanogaster*. *Genet. Res.* 24, 163–173. <https://doi.org/10.1017/s0016672300015196>
- 1114 Shearer, P.W., West, J.D., Walton, V.M., Brown, P.H., Svetec, N., Chiu, J.C., 2016. Seasonal cues
1115 induce phenotypic plasticity of *Drosophila suzukii* to enhance winter survival. *BMC Ecol.* 16, 31.
1116 <https://doi.org/10.1186/s12898-016-0070-3> PMID - 27001084
- 1117 Shimell, M., O’Connor, M.B., 2022. The cytochrome P450 Cyp6t3 is not required for ecdysone
1118 biosynthesis in *Drosophila melanogaster*. *microPublication Biology*.
1119 <https://doi.org/10.17912/MICROPUB.BIOLOGY.000611>
- 1120 Simon, A.F., Shih, C., Mack, A., Benzer, S., 2003. Steroid control of longevity in *Drosophila*
1121 *melanogaster*. *Science* 299, 1407–1410. <https://doi.org/10.1126/science.1080539>
- 1122 Škerlová, J., Lindström, H., Gonis, E., Sjödin, B., Neiers, F., Stenmark, P., Mannervik, B., 2020. Structure
1123 and steroid isomerase activity of *Drosophila* glutathione transferase E14 essential for ecdysteroid
1124 biosynthesis. *FEBS Lett.* 401, 333. <https://doi.org/10.1002/1873-3468.13718>
- 1125 Sommé-Martin, G., Colardeau, J., Beydon, P., Blais, C., Lepesant, J.A., Lafont, R., 1990. P1 gene
1126 expression in *Drosophila* larval fat body: induction by various ecdysteroids. *Arch. Insect Biochem.*
1127 *Physiol.* 15, 43–56. <https://doi.org/10.1002/arch.940150105>
- 1128 Sommé-Martin, G., Colardeau, J., Lafont, R., 1988a. Conversion of ecdysone and 20-hydroxyecdysone
1129 into 3-dehydroecdysteroids is a major pathway in third instar *Drosophila melanogaster* larvae.
1130 *Insect Biochem.* 18, 729–734. [https://doi.org/10.1016/0020-1790\(88\)90082-0](https://doi.org/10.1016/0020-1790(88)90082-0)
- 1131 Sommé-Martin, G., Colardeau, J., Lafont, R., 1988b. Metabolism and biosynthesis of ecdysteroids in the
1132 *Drosophila* development mutant *ecd1*. *Insect Biochem.* 18, 735–742.
1133 [https://doi.org/10.1016/0020-1790\(88\)90083-2](https://doi.org/10.1016/0020-1790(88)90083-2)
- 1134 Sonobe, H., Ito, Y., 2009. Phosphoconjugation and dephosphorylation reactions of steroid hormone in
1135 insects. *Mol. Cell. Endocrinol.* 307, 25–35. <https://doi.org/10.1016/j.mce.2009.03.017>
- 1136 Studinger, G., Willig, A., 1975. Biosynthesis of alpha-and beta-ecdysone in isolated abdomens of larvae
1137 of *Musca domestica*. *J. Insect Physiol.* 21, 1793–1798. [https://doi.org/10.1016/0022-1910\(75\)90243-7](https://doi.org/10.1016/0022-1910(75)90243-7)
- 1138

- 1139 Sun, W., Shen, Y.-H., Zhou, L.-X., Zhang, Z., 2016. Ecdysone Titer Determined by 3DE-3 β -Reductase
 1140 Enhances the Immune Response in the Silkworm. *The Journal of Immunology* 196, 1646–1654.
 1141 <https://doi.org/10.4049/jimmunol.1500158>
- 1142 Takeuchi, H., Rigden, D.J., Ebrahimi, B., Turner, P.C., Rees, H.H., 2005. Regulation of ecdysteroid
 1143 signalling during *Drosophila* development: identification, characterization and modelling of
 1144 ecdysone oxidase, an enzyme involved in control of ligand concentration. *Biochem. J* 389, 637–
 1145 645. <https://doi.org/10.1042/bj20050498>
- 1146 Telang, A., Frame, L., Brown, M.R., 2007. Larval feeding duration affects ecdysteroid levels and
 1147 nutritional reserves regulating pupal commitment in the yellow fever mosquito *Aedes aegypti*
 1148 (Diptera: Culicidae). *J. Exp. Biol.* 210, 854–864. <https://doi.org/10.1242/jeb.02715>
- 1149 Thompson, M.J., Feldlaufer, M.F., Lozano, R., Rees, H.H., Lusby, W.R., Svoboda, J.A., Wilzer, K.R.,
 1150 1987. Metabolism of 26-[C-14]Hydroxyecdysone 26-Phosphate in the Tobacco Hornworm,
 1151 *Manduca sexta* L., to a New Ecdysteroid Conjugate: 26-[C-14]Hydroxyecdysone 22-Glucoside.
 1152 *Arch. Insect Biochem. Physiol.* 4, 1–15. <https://doi.org/10.1002/arch.940040102>
- 1153 Thurmond, J., Goodman, J.L., Strelets, V.B., Attrill, H., Gramates, L.S., Marygold, S.J., Matthews, B.B.,
 1154 Millburn, G., Antonazzo, G., Trovisco, V., Kaufman, T.C., Calvi, B.R., FlyBase Consortium, 2019.
 1155 FlyBase 2.0: the next generation. *Nucleic Acids Res.* 47, D759–D765.
 1156 <https://doi.org/10.1093/nar/gky1003>
- 1157 Truman, J.W., Talbot, W.S., Fahrbach, S.E., Hogness, D.S., 1994. Ecdysone receptor expression in the
 1158 CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca*
 1159 development. *Development* 120, 219–234. <https://doi.org/10.1242/dev.120.1.219>
- 1160 Tu, M.-P., Yin, C.-M., Tatar, M., 2002. Impaired ovarian ecdysone synthesis of *Drosophila melanogaster*
 1161 insulin receptor mutants. *Aging Cell* 1, 158–160. <https://doi.org/10.1046/j.1474-9728.2002.00016.x>
- 1163 Uryu, O., Ou, Q., Komura-Kawa, T., Kamiyama, T., Iga, M., Syrzycka, M., Hirota, K., Kataoka, H., Honda,
 1164 B.M., King-Jones, K., Niwa, R., 2018. Cooperative Control of Ecdysone Biosynthesis in
 1165 *Drosophila* by Transcription Factors Séance, Ouija board, and Molting Defective. *Genetics* 208,
 1166 [genetics.300268.2017. https://doi.org/10.1534/genetics.117.300268](https://doi.org/10.1534/genetics.117.300268) PMID - 29187506
- 1167 Vafopoulou, X., Steel, C.G.H., Aleporou-marinou, V., Pataryas, T., 1993. Haemolymph ecdysteroid titres
 1168 at metamorphosis in the fruit fly *Ceratitis capitata*: multiple peaks not apparent in whole body
 1169 extracts. *Physiol. Entomol.* 18, 87–92. <https://doi.org/10.1111/j.1365-3032.1993.tb00453.x>
- 1170 Valentin, M., Bollenbacher, W.E., Gilbert, L.I., Kroeger, H., 1978. Alterations in ecdysone content during
 1171 the post-embryonic development of *Chironomus thummi*: correlations with chromosomal puffing.
 1172 *Z. Naturforsch. C* 33, 557–560. <https://doi.org/10.1515/znc-1978-7-818>
- 1173 Walker, E.D., Romoser, W.S., 1986. Timing of pupal-adult apolysis in mosquitoes. *J. Am. Mosq. Control*
 1174 *Assoc.* 2, 224–225.
- 1175 Walker, G.P., Denlinger, D.L., 1980. Juvenile hormone and moulting hormone titres in diapause- and non-
 1176 diapause destined flesh flies. *J. Insect Physiol.* 26, 661–664. [https://doi.org/10.1016/0022-1910\(80\)90038-4](https://doi.org/10.1016/0022-1910(80)90038-4)
- 1178 Walker, V.K., Watson, K.L., Holden, J.J.A., Steel, C.G.H., 1987. Vitellogenesis and fertility in *Drosophila*
 1179 females with low ecdysteroid titres; the L(3)3DTS mutation. *J. Insect Physiol.* 33, 137–142.
 1180 [https://doi.org/10.1016/0022-1910\(87\)90139-9](https://doi.org/10.1016/0022-1910(87)90139-9)
- 1181 Wang, J., Fan, H., Xiong, K.-C., Liu, Y.-H., 2017. Transcriptomic and metabolomic profiles of Chinese
 1182 citrus fly, *Bactrocera minax* (Diptera: Tephritidae), along with pupal development provide insight
 1183 into diapause program. *PLoS One* 12, e0181033. <https://doi.org/10.1371/journal.pone.0181033>

- 1184 Warren, J.T., Bachmann, J.S., Dai, J.-D., Gilbert, L.I., 1996. Differential incorporation of cholesterol and
1185 cholesterol derivatives into ecdysteroids by the larval ring glands and adult ovaries of *Drosophila*
1186 *melanogaster*: a putative explanation for the *l(3)ecd1* mutation. *Insect Biochem. Mol. Biol.* 26,
1187 931–943. [https://doi.org/10.1016/s0965-1748\(96\)00059-8](https://doi.org/10.1016/s0965-1748(96)00059-8) PMID - 9014338
- 1188 Warren, J.T., Petryk, A., Marqués, G., Jarcho, M., Parvy, J.-P., Dauphin-Villemant, C., O'Connor, M.B.,
1189 Gilbert, L.I., 2002. Molecular and biochemical characterization of two P450 enzymes in the
1190 ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 99,
1191 11043–11048. <https://doi.org/10.1073/pnas.162375799>
- 1192 Warren, J.T., Petryk, A., Marqués, G., Parvy, J.-P., Shinoda, T., Itoyama, K., Kobayashi, J., Jarcho, M.,
1193 Li, Y., O'Connor, M.B., Dauphin-Villemant, C., Gilbert, L.I., 2004. Phantom encodes the 25-
1194 hydroxylase of *Drosophila melanogaster* and *Bombyx mori*: a P450 enzyme critical in ecdysone
1195 biosynthesis. *Insect Biochem. Mol. Biol.* 34, 991–1010.
1196 <https://doi.org/10.1016/j.ibmb.2004.06.009>
- 1197 Warren, J.T., Yerushalmi, Y., Shimell, M.J., O'Connor, M.B., Restifo, L.L., Gilbert, L.I., 2006. Discrete
1198 pulses of molting hormone, 20-hydroxyecdysone, during late larval development of *Drosophila*
1199 *melanogaster*: Correlations with changes in gene activity. *Dev. Dyn.* 235, 315–326.
1200 <https://doi.org/10.1002/dvdy.20626>
- 1201 Webb, T.J., Powls, R., Rees, H.H., 1995. Enzymes of ecdysteroid transformation and inactivation in the
1202 midgut of the cotton leafworm, *Spodoptera littoralis*: properties and developmental profiles.
1203 *Biochem. J* 312 (Pt 2), 561–568. <https://doi.org/10.1042/bj3120561>
- 1204 Wentworth, S.L., Roberts, B., O'Connor, J.D., 1981. Ecdysteroid titres during postembryonic development
1205 of *Sarcophaga bullata* (Sarcophagidae: Diptera). *J. Insect Physiol.* 27, 435–440.
1206 [https://doi.org/10.1016/0022-1910\(81\)90093-7](https://doi.org/10.1016/0022-1910(81)90093-7)
- 1207 Whisenton, L.R., Warren, J.T., Manning, M.K., Bollenbacher, W.E., 1989. Ecdysteroid titres during pupal-
1208 adult development of *Aedes aegypti*: Basis for a sexual dimorphism in the rate of development. *J.*
1209 *Insect Physiol.* 35, 67–73. [https://doi.org/10.1016/0022-1910\(89\)90038-3](https://doi.org/10.1016/0022-1910(89)90038-3)
- 1210 Wieschaus, E., Nüsslein-Volhard, C., 2016. The Heidelberg Screen for Pattern Mutants of *Drosophila*: A
1211 Personal Account. *Annu. Rev. Cell Dev. Biol.* 32, 1–46. <https://doi.org/10.1146/annurev-cellbio-113015-023138>
- 1213 Wilson, T.G., 1982. A correlation between juvenile hormone deficiency and vitellogenic oocyte
1214 degeneration in *Drosophila melanogaster*. *Wilehm Roux Arch Dev Biol* 191, 257–263.
1215 <https://doi.org/10.1007/BF00848413>
- 1216 Wylie, H.G., 1977. Preventing and terminating pupal diapause in *Athrycia cinerea* (Diptera: Tachinidae).
1217 *Can. Entomol.* 109, 1083–1090. <https://doi.org/10.4039/Ent1091083-8>
- 1218 Xu, T., Jiang, X., Denton, D., Kumar, S., 2020. Ecdysone controlled cell and tissue deletion. *Cell Death*
1219 *Differ.* 27, 1–14. <https://doi.org/10.1038/s41418-019-0456-9>
- 1220 Yamanaka, N., Marqués, G., O'Connor, M.B., 2015. Vesicle-Mediated Steroid Hormone Secretion in
1221 *Drosophila melanogaster*. *Cell* 163, 907–919. <https://doi.org/10.1016/j.cell.2015.10.022>
- 1222 Yamanaka, N., Rewitz, K.F., O'Connor, M.B., 2013. Ecdysone Control of Developmental Transitions:
1223 Lessons from *Drosophila* Research. *Annu. Rev. Entomol.* 58, 497–516.
1224 <https://doi.org/10.1146/annurev-ento-120811-153608>
- 1225 Yasuda, T., Narahara, M., Tanaka, S., Wakamura, S., 1994. Thermal responses in the citrus fruit fly,
1226 *Dacus tsuneonis*: evidence for a pupal diapause. *Entomol. Exp. Appl.* 71, 257–261.
1227 <https://doi.org/10.1111/j.1570-7458.1994.tb01792.x>
- 1228 Yoshiyama, T., Namiki, T., Mita, K., Kataoka, H., Niwa, R., 2006. Neverland is an evolutionally conserved

- 1229 Rieske-domain protein that is essential for ecdysone synthesis and insect growth. *Development*
1230 133, 2565–2574. <https://doi.org/10.1242/dev.02428>
- 1231 Yoshiyama-Yanagawa, T., Enya, S., Shimada-Niwa, Y., Yaguchi, S., Haramoto, Y., Matsuya, T., Shiomi,
1232 K., Sasakura, Y., Takahashi, S., Asashima, M., Kataoka, H., Niwa, R., 2011. The Conserved
1233 Rieske Oxygenase DAF-36/Neverland Is a Novel Cholesterol-metabolizing Enzyme. *J. Biol.*
1234 *Chem.* 286, 25756–25762. <https://doi.org/10.1074/jbc.m111.244384> PMID - 21632547
- 1235 Žďárek, J., Denlinger, D.L., 1987. Pupal ecdysis in flies: The role of ecdysteroids in its regulation. *J.*
1236 *Insect Physiol.* 33, 123–128. [https://doi.org/10.1016/0022-1910\(87\)90084-9](https://doi.org/10.1016/0022-1910(87)90084-9)
- 1237 Zdárek, J., Denlinger, D.L., 1975. Action of ecdysoids, juvenoids, and non-hormonal agents on
1238 termination of pupal diapause in the flesh fly. *J. Insect Physiol.* 21, 1193–1202.
1239 [https://doi.org/10.1016/0022-1910\(75\)90087-6](https://doi.org/10.1016/0022-1910(75)90087-6)
- 1240 Zhang, T., Song, W., Li, Z., Qian, W., Wei, L., Yang, Y., Wang, W., Zhou, X., Meng, M., Peng, J., Xia, Q.,
1241 Perrimon, N., Cheng, D., 2018. Krüppel homolog 1 represses insect ecdysone biosynthesis by
1242 directly inhibiting the transcription of steroidogenic enzymes. *Proc. Natl. Acad. Sci. U. S. A.* 115,
1243 3960–3965. <https://doi.org/10.1073/pnas.1800435115>

1254 B) Ecdysteroid sources during *Drosophila* development, with major transitions and
1255 developmental events labelled. Known and hypothetical sources (top), with the rough
1256 20E titre from embryo to eclosion (adapted from Riddiford, 1993; Warren et al., 2006;
1257 middle), and semi-quantitative depictions of PG activity (Dai and Gilbert, 1991; Graveley
1258 et al., 2011; Redfern, 1983), *shd* expression (Graveley et al., 2011), ecdysteroid 20-
1259 monooxygenase (E20MO) activity (Mitchell and Smith, 1988) and E:20E ratio (An et al.,
1260 2017; Handler, 1982; Lavrynenko et al., 2015; Pak and Gilbert, 1987; bottom). MC,
1261 maternal conjugates. Larval and adult illustrations sourced from <https://scidraw.io>
1262 (doi.org/10.5281/zenodo.3925951; doi.org/10.5281/zenodo.4421109).

1263 **Table 1.** Occurrence of PAP-like ecdysteroid pulses, and their negative association with
 1264 pupal diapause, in species of Diptera where ecdysteroid titres during metamorphosis
 1265 have been experimentally determined

Suborder	Superfamily	Family	Species	Large ecdysteroid pulse during mid-metamorphosis	Pulse coincident with pupal–adult apolysis ^a	Lack of pulse associated with pupal diapause ^b
Brachycera	Ephydroidea	Drosophilidae	<i>Drosophila melanogaster</i>	Yes (Lavrynenko et al., 2015; Pak and Gilbert, 1987; Richards, 1981)	Yes (Bainbridge and Bownes, 1981)	NPD
Brachycera	Muscoidea	Muscidae	<i>Stomoxys calcitrans</i>	Yes (O'Neill et al., 1977)	Yes (O'Neill et al., 1977)	NPD
Brachycera	Oestroidea	Sarcophagidae	<i>Sarcophaga bullata</i>	Yes (Briers and Loof, 1980; two pulses found by Wentworth et al., 1981)	Yes (Wentworth et al., 1981)	Unknown
Brachycera	Oestroidea	Sarcophagidae	<i>Sarcophaga peregrina</i>	Yes (Moribayashi et al., 1988; Ohtaki and Takahashi, 1972)	Unknown	Pupal and pharate adult pulses absent (Moribayashi et al., 1988; Ohtaki and Takahashi, 1972)
Brachycera	Oestroidea	Sarcophagidae	<i>Sarcophaga argyrostoma</i>	Yes (two pulses found by Richard et al., 1987)	Yes (Richard et al., 1987)	First pulse smaller, second pulse absent (Richard et al., 1987)
Brachycera	Oestroidea	Sarcophagidae	<i>Sarcophaga crassipalpis</i>	Yes (Walker and Denlinger, 1980)	Yes (Walker and Denlinger, 1980)	Yes (Walker and Denlinger, 1980)
Brachycera	Oestroidea	Calliphoridae	<i>Lucilia caesar</i>	Yes (Briers et al., 1983)	Yes (Briers et al., 1983)	NPD
Brachycera	Oestroidea	Calliphoridae	<i>Protophormia terraenovae</i>	Yes (Briers et al., 1983; Gaudry et al., 2006)	Yes (Briers et al., 1983)	NPD
Brachycera	Oestroidea	Calliphoridae	<i>Calliphora vicina</i>	Yes (Briers et al., 1983)	Yes (Briers et al., 1983; Martín-Vega et al., 2017)	NPD
Brachycera	Tephritoidea	Tephritidae	<i>Ceratitis capitata</i>	Yes (Vafopoulou et al., 1993)	Yes (Vafopoulou et al., 1993)	NPD
Brachycera	Tephritoidea	Tephritidae	<i>Anastrepha suspensa</i>	Yes (Lawrence et al., 1984)	Unknown	NPD
Nematocera	Sciaroidea	Sciaridae	<i>Rhynchosciara americana</i>	Yes (Brandão et al., 2014)	Unknown	NPD
Nematocera	Chironomoidea	Chironomidae	<i>Chironomus riparius</i>	Yes (Valentin et al., 1978)	Unknown	NPD
Nematocera	Culicoidea	Culicidae	<i>Aedes aegypti</i>	Yes (Fournet et al., 1995; Margam et al., 2006;	Yes (Walker and Romoser, 1986)	NPD

				Whisenton et al., 1989)		
--	--	--	--	----------------------------	--	--

1266 ^a Pulses coincident with pupal–adult apolysis are likely to be homologous to the PAP in *Drosophila*
1267 *melanogaster*

1268 ^b NPD, no pupal diapause

1269