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Cell death during complete metamorphosis

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3 **Cell death during complete metamorphosis**
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30 **Running title:** PCD in holometabolous insects
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Abstract

In insects that undergo complete metamorphosis, cell death is essential for reshaping or removing larval tissues and organs, thus contributing to formation of the adult's body structure. In the last few decades the study of metamorphosis in Lepidoptera and Diptera has provided broad information on the tissue remodeling processes that occur during larva-pupa-adult transition and made it possible to unravel the underlying regulatory pathways. This review summarizes recent knowledge on cell death mechanisms in Lepidoptera and other holometabolous insects, highlighting similarities and differences with *Drosophila melanogaster*, and discusses the role of apoptosis and autophagy in this developmental setting.

Keywords: apoptosis, autophagy, Lepidoptera, metamorphosis, programmed cell death

1. Introduction

Both in hemimetabolous and holometabolous insects, the body plan is modified during the transition from juvenile to adult stage, but in Holometabola these modifications are marked and a pupal stage is interposed between the last larval instar and the adult stage. Insect development and metamorphosis are controlled by 20-hydroxyecdysone (20E) and juvenile hormone (JH) [1, 2]. The response triggered by these hormones in each tissue depends on receptors, activation and/or inhibition of precise pathways, and expression of specific factors that together drive the cell-specific response to the stimulus. As a consequence, according to their fate, cells and tissues during metamorphosis can: i) degenerate if they belong to specific organs present only in immature stages (e.g., head glands); ii) undergo remodeling without cells being completely replaced (e.g., fat body) or being replaced with those deriving from undifferentiated cells (e.g., midgut); or iii) generate a new adult structure thanks to the proliferation and differentiation of undifferentiated cells present in the imaginal discs (e.g., wings and legs) [3]. Although we are far from having a complete picture, in the last few decades much effort has been devoted to elucidate the mechanisms involved in the reshaping of tissues during insect metamorphosis, and programmed cell death (PCD) has emerged as a key event that drives this process.

Among the insect organs that reshape more or less extensively, or even degenerate, during the larva-pupa transition, the midgut, the fat body, head glands, and neurons are worthy of mention [Figure 1]. The larval midgut is composed of a monolayered epithelium in which columnar cells, involved in digestion and absorption of nutrients, are the most abundant cell type. When the larva enters metamorphosis, stem cells, small round cells located at the base of the monolayer, proliferate and differentiate, thus creating the midgut of the adult insect. Concomitantly, larval midgut cells are displaced by the newly forming epithelium and are pushed toward the lumen, forming a mass called the yellow body where they die [4]. The fat body is a multifunctional organ that plays a key role in nutrient storage and mobilization, but is also involved in hormonal regulation, immune response, and detoxification. At metamorphosis, tissue remodeling involves partial dissociation of clusters of cells and differentiation of primordial cells to replace those cells undergoing PCD [5]. In contrast, head glands such as the silk glands (*Bombyx mori*), salivary glands (*Drosophila melanogaster*), and labial glands (*Manduca sexta*) completely (in the first two types of head glands) or almost completely (in the latter type) degenerate during metamorphosis and no remnants of these tissues are found in the adult insect [6, 7]. Finally, some motoneurons, such as proleg motoneurons, the best studied examples of dying neurons, undergo a segment-specific pattern of PCD during larval-pupal transition together with their target muscles [8]. It must be underlined that tissue remodeling and cell death are not limited to the early phase of metamorphosis, i.e., larva-pupa transition, but can also occur at later

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3 stages, during pupal-adult transition. For example, the intersegmental muscles of moths are giant
4 syncytial fibers that begin to atrophy some days prior to adult eclosion; then, once they have played
5 their part in the emergence of the adult from the pupa, they are no longer necessary and die within a
6 few hours [9].
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10 Although tissue remodeling involving cell death processes has been reported in many species that
11 undergo complete metamorphosis, Lepidoptera and the dipteran *Drosophila melanogaster* have
12 provided the bulk of information on these processes and have made possible to unravel the underlying
13 regulatory mechanisms. Recent reviews describe in detail PCD during metamorphosis in *D.*
14 *melanogaster* tissues and organs [7, 10-12]. In contrast, a comprehensive description of the processes
15 and regulatory mechanisms involved in tissue reshaping in Lepidoptera is lacking. For this reason,
16 we report in this review the current knowledge on PCD during metamorphosis in this order, as well
17 as in other holometabolous and hemimetabolous insects, highlighting similarities and differences
18 with *D. melanogaster*.
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26 27 **2. Programmed cell death**

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29 PCD plays a fundamental role in the normal development of metazoans as is essential for removing
30 damaged cells and for remodeling and sculpting tissues during morphogenesis. Many different forms
31 of cell death, sometimes characterized by overlapping features, have been reported in the literature.
32 Since the definition and the interpretation of the various cell death processes are complex and novel
33 mechanisms that regulate cell death pathways are continuously being unveiled, the Nomenclature
34 Committee on Cell Death has formulated detailed guidelines (see [13] for the last version). However,
35 two cell death processes have been mainly described during development, i.e., apoptosis and
36 autophagic cell death. While the biochemical pathways that regulate apoptosis have been widely
37 studied, less information about the mechanisms and genes regulating autophagic cell death is
38 available [13, 14].
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46 Apoptosis is a caspase-dependent cell death process characterized by DNA fragmentation,
47 cytoplasmic blebbing, and engulfment and degradation of the apoptotic bodies by phagocytes.
48 Caspases are cysteine proteases that include two groups of enzymes: initiator caspases are triggered
49 by the apoptotic stimulus and, in turn, activate effector caspases that cleave the majority of cell
50 components and lead to the death of the cell [15]. The apoptotic pathway has been well characterized
51 in *Drosophila* where it involves the intervention of seven caspases that show enzymatic function
52 comparable to that characterized in *Caenorhabditis elegans* and mammals, although the regulation of
53 apoptotic machinery is slightly different in this insect. Among the major differences, it is worth
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3 highlighting that in *Drosophila* cytochrome *c* is not required for the assembly of the apoptosome and
4 caspases are mainly regulated by inhibitor of apoptosis proteins (IAPs) [16].

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6 On the other hand, cells that undergo autophagic cell death are capable of digesting their own contents
7 without the aid of engulfing phagocytes. The morphological hallmark of autophagic cell death is the
8 formation and the presence of autophagosomes in the dying cell. However, this does not necessarily
9 mean that autophagy plays an active role in the death of the cell [14]. Autophagy is a catabolic process
10 that leads to the degradation of proteins and organelles through their engulfment into the
11 autophagosome, a double membrane structure which is formed from an isolation membrane whose
12 origin is under debate. Once the autophagosome fuses with lysosomes, the inner content is degraded
13 and the breakdown products are recycled back into the cytosol. The autophagic pathway is highly
14 conserved among eukaryotes and is mediated by more than 35 AuTophagy-related (*ATG*) genes,
15 originally identified from genetic screening for yeast mutants unable to survive under starvation.
16 Autophagosome formation is triggered by activating the Atg1 kinase complex, which is obtained by
17 relieving the inhibitory action of Target of rapamycin (Tor). The Atg6 complex then activates
18 nucleation of the isolation membrane, while the elongation and expansion of the autophagosome
19 membrane to form a closed autophagosome depends on two conserved ubiquitin-like conjugation
20 systems, i.e., the Atg12-Atg5/Atg16 complex and Atg8, which mediate the lipidation and insertion
21 of Atg8 in the growing autophagosome membrane [17].

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23 In some developmental settings a third type of cell death has been observed, i.e., necrosis, which is
24 characterized by an increase in cell volume, swelling of cytoplasmic organelles, membrane rupture,
25 and release of cytoplasmic content in the extracellular environment. Although necrosis is usually
26 considered an uncontrolled form of cell death, its execution may be finely regulated at least in some
27 contexts (see for example necroptosis) [13] [Figure 2].

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29 It must be highlighted that the role of autophagy in tissue remodeling during insect metamorphosis is
30 still widely debated, and, over the years, a copresence of autophagic and apoptotic features in insect
31 organs during metamorphosis has been described (for an overview on the early age of autophagic and
32 apoptotic studies in holometabolous insects, see [18-20]). Moreover, recent studies suggest an
33 intimate relationship between the two processes and corroborate the hypothesis that they both have
34 an active role in remodeling/removing larval tissues.

35 36 37 **3. Programmed cell death in Lepidoptera**

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39 Lepidoptera, and in particular *B. mori*, *Helicoverpa armigera*, and *M. sexta*, have been used in the
40 last two decades to gain insight into the endocrinological and physiological aspects of cell death
41 processes in insects. More recently, thanks to the development of genetic tools such as RNAi and
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3 CRISPR-Cas9, the first two species have also provided information on the regulatory mechanisms of
4 cell death. Although knowledge on cell death in *Drosophila* might be exploited for studying the role
5 of autophagy and apoptosis in normal and pathological conditions, it is worth underscoring the fact
6 that some of the most important insect pest species belong to Lepidoptera and, therefore, an
7 understanding of the processes that intervene during metamorphosis and identifying critical
8 regulatory factors involved in this crucial process could be useful for application purposes.
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15 3.1 The apoptotic process in Lepidoptera

16 Caspase-1 represents the first caspase found in Lepidoptera. It was identified and characterized in
17 *Spodoptera frugiperda* where it acts as the main effector caspase and is able to trigger apoptosis. The
18 activation process requires cleavage of the p37 proenzyme in a two-step process to produce the p12
19 and p19 subunits [21]. Thereafter, homologs, characterized by conserved amino acid sequences and
20 similar mechanisms of action, were found in *Spodoptera littoralis* (SlCaspase-1) [22], *B. mori*
21 (BmCaspase-1) [23], and *Galleria mellonella* (GmCaspase-1) [24]. The complexity of the apoptotic
22 pathway in Lepidoptera was brought to light thanks to a genome-wide analysis of apoptosis-related
23 genes in *B. mori* that identified two initiator caspases, namely BmDronc and BmDreed, and three
24 effector caspases, BmCaspase- N, BmCaspase-1, and BmICE [25]. Moreover, a number of homologs
25 of both human and *Drosophila* apoptosis-related genes were reported and the presence of both the
26 extrinsic and the intrinsic apoptotic pathway (i.e., initiated by extracellular and intracellular stimuli,
27 respectively), described in other organisms, was also hypothesized for the silkworm [25].
28 Subsequently, an extensive survey of lepidopteran-derived EST datasets in which sequences from 27
29 different lepidopteran species were compared to *Drosophila* homologs, helped identify 63 putative
30 caspase genes in Lepidoptera [26]. These caspases clustered into six distinct groups: caspase-1, -2,
31 and -3 were considered homologs to *Drosophila* Dcp-1, Drice, and Decay, respectively, and thus
32 classified as effector caspases due to their short prodomain; caspase-5 and -6 showed structural
33 features typical of initiator caspases and shared similarities with *Drosophila* Dronc and Dreed,
34 respectively; the function of caspase-4 as effector or initiator caspase remained uncertain.
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36 20E represents one of the major regulators of apoptosis in Lepidoptera [27]. As in *Drosophila*, upon
37 binding to its heterodimeric nuclear receptor complex, which consists of the ecdysone receptor (EcR)
38 and ultraspiracle (USP), this hormone elicits the transcription of early and late genes and the
39 activation of apoptosis. Accordingly, RNAi-mediated knockdown of USP decreases the expression
40 of apoptosis-related genes and reduces apoptosis in *B. mori* larval fat body [27]. However, regulation
41 of apoptosis by 20E in Lepidoptera may be more complex than expected. In fact, based on studies
42 performed in the anterior silk gland of *B. mori*, it has been proposed that cell death is regulated by a
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3 double pulse of 20E, which peaks twice during larval-pupal transition [28]. In particular, the first
4 ecdysone peak (named commitment peak) drives a genomic response by upregulating the expression
5 of apoptosis-related genes. This effect is mediated by the binding of 20E to the EcR/USP receptor
6 complex. A second pulse of hormone (the so-called metamorphic peak) occurs later, at pupal stage,
7 and is much stronger than the commitment peak. The effects of this second wave of ecdysone are
8 instead mediated by a putative ecdysone membrane receptor, probably a G protein-coupled receptor
9 (GPCR), which activates effector caspases and triggers apoptosis (nongenomic response) [28]. The
10 involvement of GPCRs in mediating 20E signaling that leads to apoptosis has been reported in *H.*
11 *armigera* as well [29]. Similar to the silk gland, a biphasic activation of the apoptotic process by 20E
12 has also been observed in the silkworm midgut. In particular, Romanelli et al. [30] demonstrated that
13 the first ecdysone peak promotes the transcriptional upregulation of apoptotic genes in the midgut,
14 but a second 20E peak is necessary to obtain caspase cleavage and the subsequent activation of the
15 apoptotic process. In this setting, IAPs, which act through binding to key apoptotic players and whose
16 removal is necessary for caspase activation, could contribute to the regulation of the apoptotic process
17 by 20E. In Lepidoptera, IAPs are highly expressed at the end of the last larval stage when the 20E
18 commitment peak occurs [30-35] and the high levels of IAPs can likely block the activation of
19 caspases. Conversely, after metamorphosis begins, and concomitantly with the occurrence of the 20E
20 metamorphic peak, the expression of IAPs decreases, while the mRNA levels of death activator genes
21 and caspase reach a maximum, a clear indication of the shift of the cells to the apoptotic condition.
22 This evidence confirms the existence of a cross-link between IAPs and caspases in regulating the
23 activation of apoptosis in Lepidoptera and suggests that these anti-apoptotic proteins may play a role
24 in the two-step process needed to activate apoptosis following the 20E peaks. Recently, the Hippo
25 signaling pathway, which controls the organ size by restricting cell proliferation and promoting
26 apoptosis, was shown to be involved in regulating cell death through IAP [36]. Hippo is highly
27 expressed during metamorphosis by 20E induction to regulate the activity of the transcriptional
28 coactivator Yorkie. In particular, following 20E stimulation, Yorkie is phosphorylated and retained
29 in the cytoplasm as it interacts with the adaptor protein 14-3-3-ε. The cytoplasmic localization of
30 Yorkie suppresses the transcriptional activation of IAP and releases its inhibitory effects on caspases,
31 thus leading to apoptosis [36, 37]. Interestingly, He and colleagues [38] showed that IAPs can
32 intervene at later stages of metamorphosis as well. In fact, IAP survivin can preserve both the
33 imaginal midgut that forms at late pupal stage and the imaginal fat body from cell death, thus allowing
34 the midgut and fat body to develop in the adult moth. This evidence demonstrates that, similarly to
35 *Drosophila*, IAPs are the main regulators of cell survival in Lepidoptera and prevent the inappropriate
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3 activation of caspases during development, and that various IAPs may play specific, distinct roles at
4 different developmental stages.
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6 To fully understand the regulatory process of apoptosis, JH must also be considered. In fact, studies
7 based on the administration of methoprene, a JH analog, indicate that JH plays an inhibitory role
8 towards apoptosis in many tissues [33]. In particular, the anterior silk glands become competent to
9 respond to 20E before the prepupal period and the commitment to die is preceded by a loss of
10 sensitivity to JH [3]. Moreover, Wang et al. [39] demonstrated that the counteracting activity of 20E
11 and JH in inducing cell death is mediated by different levels of calcium mobilization. In fact, upon
12 binding to GPCRs, 20E induces high calcium levels in the cell activating caspases and inducing
13 apoptosis, while lower calcium levels, induced by JH, have the opposite effect.
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22 3.2 *The autophagic process in Lepidoptera*

23 A genome-wide search of autophagy-related genes performed in 2009 paved the way for the
24 molecular analysis of the autophagic process in Lepidoptera [40]. Homologs of several Atg proteins
25 as well as factors involved in the PI3K signal transduction pathway were soon identified, thus
26 demonstrating the existence of a well-organized autophagic pathway in these insects [19, 30, 40-44].
27 Zhou et al. [45] also identified two paralogous genes that code for Tor, a key regulatory factor of
28 autophagy in eukaryotes. The upregulation of both *BmTOR* genes by starvation and 20E, two signals
29 that promote autophagy in *Drosophila* by recruiting Tor, confirms that silkworm Tor is able to
30 mediate the activation of the autophagic pathway triggered by nutrient conditions and hormones [45].
31 The autophagic pathway in the fat body is elicited by 20E through a twofold activation process. In
32 fact, the 20E commitment peak during larval-pupal transition inhibits the PI3K/Torc1 (Target of
33 rapamycin complex 1) pathway and activates the downstream Atg1/Atg13 complex, thus inducing
34 autophagosome initiation. In parallel, 20E also contributes to autophagosome formation by
35 upregulating the transcription of *ATG* genes: this effect is mediated by the action of Broad-Complex
36 (Br-C) and downstream proteins, but also through direct interaction with the promoter of *ATG* genes,
37 as demonstrated for *BmATG1* [46]. Data on the midgut support the double regulatory effects of 20E
38 on autophagy in lepidopteran organs. In fact, a single dose of ecdysone in this tissue can induce the
39 transcription of *ATG* genes and, at the same time, activate the autophagic flux through Torc1
40 inhibition [30]. By using loss-of-function and gain-of-function approaches, it has been recently
41 demonstrated that not only ecdysone biosynthesis but also 20E depletion is fundamental for
42 regulating autophagy-mediated tissue degeneration on the silkworm. In fact, Li et al. [47] showed
43 that the overexpression of ecdysone oxidase, a 20E inactivation enzyme, specifically inhibits
44 autophagy and impairs the degeneration of middle silk gland, confirming that 20E-triggered
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3 autophagy is required for tissue histolysis in the silkworm. In addition, the authors speculate that the
4 tissue-specific differences in response to ecdysteroid, such as timing of autophagy and apoptosis
5 activation, may be partially mediated by a differential expression of ecdysteroid inactivation
6 enzymes.
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10 Only limited information on the signaling pathway that links autophagy to JH is available for
11 Lepidoptera. In this context, it has been shown that administration of JH to *Mamestra brassicae*
12 during last larval instar inhibits autophagy in the fat body [48]. Moreover, RNAi of the putative JH
13 receptor Met has a marked effect on the autophagic response during metamorphosis in *B. mori* [49].
14 This evidence suggests that autophagy is inhibited by JH and it can be activated at metamorphosis by
15 an increase in 20E concentration, when the JH titer is low.
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22 3.3. Apoptosis and autophagy in Lepidoptera: a matter of coexistence, cooperation, or overlap?

23 Coexistence of autophagic and apoptotic features within the same tissue or organ has been reported
24 in both Lepidoptera [6, 31, 50, 51] and *Drosophila* (for a review see [7]). Research in these insects
25 has been mainly devoted to unraveling the role of the two processes and to looking for potential
26 factors that mediate the interaction between them. By using specific markers, it has been
27 demonstrated that, in general, autophagy is activated in many lepidopteran organs at wandering stage
28 and precedes apoptosis during metamorphosis [6, 30, 31, 34]. As a double pulse of ecdysone is needed
29 to activate apoptosis, as described above, the occurrence of apoptosis follows that of autophagy, but
30 the precise timing of activation is organ-specific and is related to the role played by autophagy as
31 follows. In the posterior silk gland of *B. mori*, apoptosis is activated 48-72 hours after the autophagic
32 process. In this tissue, glycogen stores need to be mobilized and the cytoplasmic components
33 degraded by autophagy to produce the energy that the gland cells need to stay alive and complete the
34 spinning of the cocoon. Conversely, apoptosis leads cells to death once the silk gland is no longer
35 useful to the larva and apoptotic bodies can be removed by phagocytes in the larval hemocoel [6]. In
36 the larval fat body the temporal shift in the occurrence of autophagy and apoptosis is not as evident.
37 However, the two processes seem to have different roles: autophagy is activated during
38 metamorphosis to promote lipolysis and trophically support the insect [46], while apoptosis is
39 necessary for remodeling this tissue [27, 52]. Thus, the evidence collected seems to exclude an
40 involvement of autophagy in the death of fat body and silk gland cells. The function of autophagy
41 and apoptosis has been investigated in the silkworm midgut, too. In this setting, the administration of
42 autophagy inhibitors to the larvae leads to an increased degeneration of the larval midgut compared
43 to the control, thus suggesting a prosurvival role of autophagy in this tissue. Moreover, caspase
44 inhibitors significantly delay larval midgut degeneration, thus demonstrating that apoptosis is
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3 sufficient to drive this process during metamorphosis [30]. The precise role of autophagy in this
4 scenario can be appreciated by considering its intimate relationship with apoptosis and necrosis. In
5 fact, autophagy is activated in larval midgut cells as soon as the larva stops feeding at the beginning
6 of metamorphosis. This is initially necessary to cope with nutrient starvation, but the larval cells are
7 then digested completely. Meanwhile, cells undergo apoptosis and, subsequently, secondary necrosis
8 determines the release of the breakdown products, generated by the autophagic process, in the
9 extracellular environment (i.e., the midgut lumen). These products can be directly absorbed by the
10 surrounding cells that, in the meantime, are forming the new midgut epithelium of the adult [Figure
11 3]. Thus, this tissue can recycle products deriving from the degeneration of larval midgut cells that
12 contribute in this manner to trophically sustain the silkworm [4, 53]. Therefore, as in fat body [46]
13 and silk gland [6], autophagy has a prosurvival role in the larval midgut, while apoptosis is the major
14 process responsible for the death of the larval cells, questioning the existence of autophagic cell death
15 in Lepidoptera. This setting in which autophagy guarantees cell survival, apoptosis plays a key
16 function in driving the demise of larval tissues, and cell death processes are caspase-dependent, is
17 well supported by functional experiments in which the use of inhibitors [30] and gene silencing by
18 RNAi [36, 54, 55] have been used to block the autophagic and the apoptotic pathway in the larval
19 organs of *B. mori* and *H. armigera*. However, at least four different, alternative scenarios that confer
20 further complexity to developmental cell death in Lepidoptera have been described in *M. sexta* and
21 other insects belonging to this order. Importantly, though, some of them still require further
22 investigation using functional or genetic tools:

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1) in dying larval motoneurons, which exhibit an autophagic phenotype without features
characteristic of apoptosis, the administration of caspase inhibitors impairs the cell death process
[56], suggesting that caspase-dependent autophagic cell death occurs [8]; 2) since the early studies of
Lockshin [57-60], it has been evident that the death process causing intersegmental muscles to
disappear soon after adult eclosion displays typical autophagic features and does not show any
apoptotic marker such as DNA condensation and fragmentation or caspase activation. According to
this evidence, the term "programmed cell death" was introduced and this degenerative process
classified as autophagic cell death [57, 58]. The removal of these cells is indeed mediated by
lysosomal enzymes as well as by proteasomal activity, as demonstrated by the upregulation of several
genes associated with proteolysis, and increased protein catabolism through the ubiquitin-proteasome
pathway [61-64]. This cell death scenario likely reflects the impossibility for these muscles to be
phagocytosed due to their large size. For this reason, they undergo autophagic cell death which not
only removes unnecessary larval cells in the moth, but also provides amino acids that can serve as a
nutrient source for the adult insect [9]; 3) similarly to intersegmental muscles, the involution of labial

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3 glands involves autophagy-associated programmed cell death without any sign of apoptotic features
4 [65], although the apoptosis machinery is conserved and functional in this organ [66]. In this case,
5 caspase activity is substituted by the proteolytic activity of lysosomal cathepsins [66]; 4) a recent
6 view identified cathepsins as the cell death switch. Cathepsins are cysteine or aspartate proteases that,
7 in Lepidoptera, play several roles in regulating metamorphic and cell death processes (for a review,
8 see [67]). The expression of cathepsin B, D, and L is induced by 20E and a correlation between their
9 expression and degradation of larval midgut and fat body has been established [68-70]. A recent study
10 clearly demonstrates that cathepsin L is able to cleave procaspase 1 into the mature peptide and
11 activate apoptosis in *H. armigera* midgut [71]. In addition, cathepsin L knockdown in *Antheraea*
12 *pernyi* decreases the expression of several apoptotic genes and impairs fat body dissociation,
13 confirming that this cathepsin is located upstream of the apoptotic pathway [72]. Together, this
14 evidence indicates that cathepsins play a major role in triggering apoptosis.

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16 In conclusion, the contrasting data obtained so far suggest that the involvement of caspases in PCD
17 could be context-dependent in Lepidoptera. These results also leave the role of autophagy in the
18 metamorphosis of these holometabolous insects still unresolved, and foreshadow a more complex
19 picture of cell death processes than expected.

3.4 Autophagy and autophagic cell death: learning from *Drosophila*

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21 In contrast to Lepidoptera, strong evidence demonstrating an active role of autophagy in ecdysone-
22 mediated PCD in specific tissues comes from *Drosophila*. Apoptosis and autophagy must act in
23 parallel to remove salivary glands during metamorphosis. Following puparium formation, the high
24 titer of 20E triggers the expression of genes that regulate both mechanisms. The impairment of one
25 of these two processes results in partial salivary glands degradation, while only the combined
26 inhibition of both processes can significantly delay their removal [73]. Salivary glands can be
27 considered a unique organ in which both apoptosis and autophagy cooperate to trigger cell death,
28 although the involvement of other cell death pathways cannot be excluded as organ removal is not
29 completely blocked when the two mechanisms are inhibited [74]. In the *Drosophila* larval midgut,
30 although apoptosis is activated during PCD, autophagy is the process that causes cell demise. It must
31 be highlighted that caspase activation occurs in the absence of the main initiator and effector caspases
32 (i.e., Ark, Dronc, Drice, Dcp-1, and Strica) and the primary contributor of caspase activity in dying
33 midgut is Decay [75], which is, however, not essential for PCD in this tissue [75]. Therefore, the
34 canonical apoptotic pathway is not required for larval midgut removal. In contrast, inhibition of
35 autophagy by using *ATG* loss-of-function mutants (including *ATG1*, *ATG2*, and *ATG18*) induces a
36 severe delay in the tissue remodeling without a decrease in caspase activity [75], indicating a key role
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3 for autophagy in midgut cell death. More recently, different components of the autophagic machinery
4 that are required during midgut removal have been identified and their role defined [76], but further
5 studies are required to fully delineate the regulation and mechanisms that mediate autophagy-
6
7 dependent cell death in the midgut. Interestingly, a comparison of the components of the autophagic
8 pathway essential for midgut degradation and those involved in starvation-induced autophagy in the
9 fat body reveals that only a subset of *ATG* genes involved in the latter are required for autophagy-
10 dependent cell death in the midgut [10], indicating that the regulative pathways for the autophagic
11 process may vary in the two settings. *Drosophila* fat body during metamorphosis is extensively
12 remodeled by a balanced 20E-dependent activation of apoptosis and autophagy. The 20E primary
13 response gene *E93* plays a key role in the activation of both pathways [77], thanks to the inhibition
14 of PI3K-Torc1 pathway, but the precise role of apoptosis and autophagy in this organ is not
15 completely understood.

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17 Taken together, these data demonstrate that, during insect metamorphosis, apoptosis and autophagy
18 play a key role in tissue remodeling, but their direct involvement in cell demise is highly tissue-,
19 context-, and species-specific; moreover, it is still unknown what determines whether autophagy has
20 a prosurvival or prodeath function in this setting. *Drosophila* has proven to be a powerful model for
21 studying regulative processes and understanding gene function in insects, but the knowledge derived
22 from this insect cannot be extended to other species without the risk of missing important
23 peculiarities. PCD in insect metamorphosis represents a perfect example of this concept. A
24 comparative approach as well as functional studies and the use of large-scale screenings to evaluate
25 the expression profile of autophagic and apoptotic genes in different insects and tissues will hopefully
26 clarify the precise role of the pathways activated for reshaping or removing larval organs during
27 metamorphosis and unravel why PCD is executed via different mechanisms in the same organ of
28 insects belonging to diverse, or even to the same, orders. Moreover, great attention must be devoted
29 to possible interactions among different PCD mechanisms. Some apparently contrasting results
30 reported in the literature, which are still not completely understood (e.g., the different roles played
31 by autophagy and apoptosis in tissue remodeling among different lepidopteran species, and between
32 them and *Drosophila*), may derive from different experimental approaches, but may also reflect a
33 real variability that depends on factors whose meaning is not completely clear or still need to be
34 discovered. Indeed, we are still far from having exhaustive and complete knowledge of the complex
35 pathways that regulate insect body remodeling and only after the genes and pathways activated in
36 different tissues/organs/insects have been characterized in depth we will understand why different
37 PCD mechanisms are set in motion in a context-specific manner during metamorphosis.

3.5 Cross-talk between autophagy and apoptosis

In the search for potential cross-talk between autophagy and apoptosis in Lepidoptera, considerable effort has been devoted to identifying potential mediators between the two processes.

Different Atg proteins have been suggested as molecular links between autophagy and apoptosis in Lepidoptera. In *B. mori* cell lines, it was recently proposed that the induction of apoptosis, which follows an autophagic response triggered by 20E or starvation, could be mediated by BmAtg5 and BmAtg6 after their cleavage by calpains [78, 79]. Although the association of cleaved-BmAtg5 to anti-apoptotic Bcl-2 family proteins to trigger cytochrome *c* release from mitochondria and caspase activation, similarly to that observed in mammals, has not been investigated yet, this mechanism could explain why 20E and/or starvation cause cell death in the larval organs of Lepidoptera during metamorphosis, with autophagy preceding apoptosis [79]. Support for this hypothesis was obtained in *H. armigera*, where it was shown that the cleavage of Atg5 could promote the switch from autophagy to apoptosis in the larval midgut [54]. In particular, Atg5 mediates the activation of autophagy triggered by low levels of 20E, thus leading to the survival of the cell. High levels of ecdysone determine an increase in intracellular calcium levels, which results in the cleavage of Atg5, probably mediated by calpains, and finally to the activation of apoptosis, causing the cell to die. A subsequent study, focused on the Atg12-Atg5 complex, which is required for Atg8 lipidation, demonstrated that a low concentration of 20E can promote the conjugation of Atg5 to Atg12, thus activating autophagy. Conversely, higher levels of the hormone induce the cleavage of Atg5 and impair the Atg5-Atg12 conjugation process, thus switching to apoptosis [55].

A final consideration concerns the PI3K/Akt pathway, which plays an important role in regulating a number of cellular processes, such as cell proliferation, cell survival, cell death, signal transduction, and protein synthesis, and which may also deserve attention as a potential point of interaction between autophagy and apoptosis. This signaling pathway in fact represents the mediator that leads to the activation of autophagy at metamorphosis via nutrient signals and hormones, at least in the larval fat body [5]. Furthermore, a recent study has reported that the expression of apoptosis-related genes, such as caspases, during the spinning period is regulated by PI3K/Akt, suggesting a possible role of this signaling pathway in activating apoptosis in the posterior silk gland of *B. mori*, too [80]. However, further investigation is needed to confirm this hypothesis for the PI3K/Akt pathway and, hopefully, to characterize the putative regulatory mechanism.

4. Cell death in other holometabolous insects

In addition to Lepidoptera and *Drosophila*, the involvement of cell death processes in tissue remodeling during metamorphosis has been reported in Coleoptera, Hymenoptera, and other Diptera.

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3 Since in most cases only morphological studies were carried out and there is no general agreement
4 on the features that are typical of the different cell death forms, it is difficult to get a clear picture
5 even in species that belong to the same insect order. Moreover, only limited functional evidence has
6 been provided to evaluate the role of cell death processes in this setting. **In spite of the fact that
7 information is fragmentary and that further considerations of the regulatory mechanisms are not
8 possible, we included an overview of other Holometabola for the sake of completeness.**
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11 In the honeybee, *Apis mellifera*, the intervention of PCD has been described in many developmental
12 processes [81], not strictly related to metamorphosis. During larva-pupa transition, a concomitant
13 activation of autophagy and apoptosis is necessary for the removal of the larval midgut [82], the
14 hindgut [83], salivary glands [84, 85], and Malpighian tubules [86]. In particular, in the salivary
15 glands, autophagy and apoptosis act in a coordinated manner to reduce the thickness of the larval
16 epithelium by releasing cytoplasmic protrusions from the apical side of the cells. This process leads
17 to the progressive degeneration of the epithelium and the authors speculate that the remnants of the
18 glands are recycled back into the hemolymph without the intervention of phagocytes [85]. Both
19 autophagy and apoptosis are responsible for the removal of the larval midgut and fat body in another
20 hymenopteran, the stingless bee *Melipona quadrifasciata* [87, 88]. In the fat body of this insect,
21 autophagy appears to be the predominant process, while only few apoptotic cells can be observed:
22 this pattern could be explained by considering that this larval tissue is not subjected to significant
23 destruction during metamorphosis, but it is only remodeled to generate the adult fat body.
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26 In hematophagous Diptera, the remodeling of the midgut during metamorphosis is fundamental to
27 produce an organ that is able to cope with the change in dietary requirements between the larval and
28 the adult stage. Malta et al. [89] demonstrated that metamorphic changes in the larval midgut
29 epithelium of sand flies are associated with the upregulation of several *ATG* genes, due to the presence
30 of ecdysone responsive elements in their promoter, as occurs in the silkworm. This evidence confirms
31 that the mechanisms controlling the onset of metamorphosis are conserved across multiple orders of
32 holometabolous insects. In *Aedes aegyptii*, it has been demonstrated that the differential expression
33 of EcR and USP isoforms, as well as transcription factors, allows 20E to finely regulate cell death in
34 the midgut [90]. A precise regulation of the death processes by 20E has been confirmed in
35 *Rynchosciara americana* [91]. In this case, the prepupal and pupal ecdysone peak seem to trigger
36 salivary gland histolysis and fat body reorganization, respectively. Finally, Mittapalli and Shukle [92]
37 showed that the anti-apoptotic factor DAD1 (defender against apoptotic cell death) is highly
38 expressed up to pupation in order to inhibit unwanted cell death events prior to metamorphosis. This
39 evidence confers further complexity to the mechanisms that inhibit apoptosis in insects, in addition
40 to the involvement of IAPs, as discussed above for Lepidoptera.
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3 Studies in Coleoptera are mainly limited to the mealworm, *Tenebrio molitor*. In this coleopteron many
4 *ATG* genes have been cloned and characterized and, despite some variations, they are expressed in
5 various tissues during development [93-95]. In particular, *ATG8* is highly expressed in the midgut at
6 pupal stages, corroborating a role of autophagy in the remodeling process of this tissue. It is
7 noteworthy that *ATG5* silencing impairs Atg8 lipidation [94], thus demonstrating not only the
8 presence of autophagy-related homologs in this insect, but also the high conservation of the whole
9 autophagy machinery. Parthasarathy and Palli [96] demonstrated that cell death, essentially apoptosis,
10 in the *Tribolium castaneum* midgut is tightly controlled by 20E, which triggers a cascade of early and
11 late genes as in *Drosophila*, and that these effects are counteracted by JH. A peculiar structure that is
12 remodeled during metamorphosis has been reported in horned beetles [97]. Many species present
13 horns, namely, cuticular projection of the head and/or prothorax that are completely degraded once
14 they have facilitated the shedding of the head capsule during the pupal stage, or are remodeled to
15 generate interindividual diversity at the adult stage. This remodeling process of the horn ensues after
16 a classical apoptotic pathway is activated, as demonstrated by a microarray screening. Thus, in this
17 case, the metamorphic remodeling of a body structure is not associated with any physiological
18 function, but is needed to generate morphological diversity within and between species.
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32 **5. Tissue remodelling in hemimetabolous insects and evolutionary perspectives**

33 In hemimetabolous insects the morphological changes occurring during the transition from juvenile
34 (i.e., nymph) to adult stage are less extensive than in Holometabola. These apparently less dramatic
35 changes probably explain why relatively little attention has been devoted to the mechanisms and
36 pathways involved in the remodeling and removal of tissues and organs during this event of the life
37 cycle of Hemimetabola compared to Holometabola. Nonetheless, considering the relevance of cell
38 death in the metamorphosis in Holometabola, the characterization of the molecular pathways
39 involved in remodeling and removing tissue during the transition from juvenile to adult stage in
40 Hemimetabola can aid our understanding of how metamorphosis has evolved from less to more
41 derived insect species.
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49 Since the studies of Wigglesworth [98, 99] on *Rhodnius prolixus* it has been clear that the onset of
50 metamorphosis is hormonally regulated. Later, it was suggested that hormone-dependent
51 programmed cell death is involved in restructuring the metathoracic pleuroaxillary muscles in
52 *Locusta migratoria* immediately after the imaginal molt [100], but functional studies were not
53 performed until more recently. By using RNAi, activators and inhibitor proteins that coordinate the
54 degeneration of the prothoracic glands during the onset of the adult stage of *Blattella germanica* have
55 been identified [101]. The inhibitor of apoptosis BgIAP1 is required to block premature cell death
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3 processes in this gland, suggesting that the mechanism involved in impairing inappropriate caspase
4 activation during juvenile development is highly conserved in insects. The degeneration of
5 prothoracic glands depends on the complex cascade triggered by 20E that converges on the activation
6 of the nuclear receptor BgFtz-f1. BgE75 acts as a suppressor of apoptotic cell death by restricting the
7 expression of BgFtz-f1 to the end of the last nymphal instar. Moreover, JH plays a key role in
8 preventing prothoracic glands degeneration during nymphal development, probably exerting its effect
9 directly on the ecdysteroid-dependent transcriptional cascade, because treating last instar nymphs
10 with the JH analog methoprene reduces the expression of *FTZ-F1* and prevents degeneration of the
11 gland. After this first functional study, efforts were devoted to identifying the early genes activated
12 by JH and 20E during incomplete metamorphosis [102-104] and the event that triggers the decrease
13 of JH at the beginning of the last nymphal instar [105]. In contrast, less attention has been dedicated
14 to PCD in postembryonic development. The evidence obtained so far indicates that the pathways
15 triggering metamorphosis are generally conserved, although some differences have been shown.
16 Regarding the regulation of JH level in the hemolymph, which always remains low during the last
17 nymphal instar, myoglianin plays a key role [105]. In Holometabola, myoglianin is not involved in
18 regulating JH production, probably because this regulatory mechanism might not be appropriate to
19 determine a rapid pattern of decrease-increase-decrease of JH production, typical of the prepupal
20 stage in Holometabola. Another interesting peculiarity of Hemimetabola concerns *Br-C* [102]. *BR-C*
21 is an early response gene in the 20E signaling pathway whose expression is also influenced by JH,
22 but in a different manner in holometabolous and hemimetabolous insects. In the latter, JH stimulates
23 *BR-C* transcription from the beginning to the end of the nymphal stages [106, 107] and *Br-C* seems
24 to be involved in regulating wing primordia development during nymphal stages [106, 107]. In
25 contrast, JH inhibits *BR-C* expression in holometabolous insects during the juvenile stage and the
26 transcript levels of this gene increase at the end of the last larval instar, in correspondence to the
27 commitment peak, and remain high during the prepupal phase, triggering entrance to the pupal-adult
28 stage [108, 109]. Moreover, the evidence that the expression patterns triggering adult morphogenesis
29 (i.e., decrease in *KR-H1* transcripts and increase in *E93* expression) occur at the beginning of the last
30 instar nymph in Hemimetabola and in the prepupae of Holometabola suggests that the last nymphal
31 instar of hemimetabolous insects is homologous to the holometabolous pupa [102].
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33 Some questions remain open. Data reported in the literature seem to indicate, at least in the tissues
34 examined so far, that apoptosis is the mechanism involved in restructuring the body plan during
35 metamorphosis in Hemimetabola. Autophagic cell death has never been described, but its
36 involvement cannot be excluded a priori. Is autophagic cell death present only in insects undergoing
37 complete metamorphosis? If so, how relevant is this evidence from an evolutionary point of view?
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3 The answers to these questions will shed light on the evolution of metamorphosis and processes that
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Figure legends

Figure 1. Remodeling of silkworm organs during metamorphosis. (a,e) Stereomicroscopic image showing the internal anatomy of *B. mori*; (b,f) cross-sections of the fat body; (c,g) cross-sections of the silk gland; (d,h) cross-sections of the midgut. E: larval midgut epithelium; F: fat body; Y: yellow body; L: midgut lumen; M: midgut; P: pupal midgut epithelium; arrowheads: silk glands; arrows: silk gland epithelium; asterisk: silk gland lumen.

Bars: 25 mm (a,e); 50 μm (b,f); 200 μm (c); 100 μm (d,g,h)

Figure 2. Schematic representation of the apoptotic, necrotic, and autophagic processes.

Figure 3. Schematic representation of the remodeling process that occurs in silkworm midgut during metamorphosis. Larval epithelium is indicated in orange, pupal-adult epithelium in yellow.

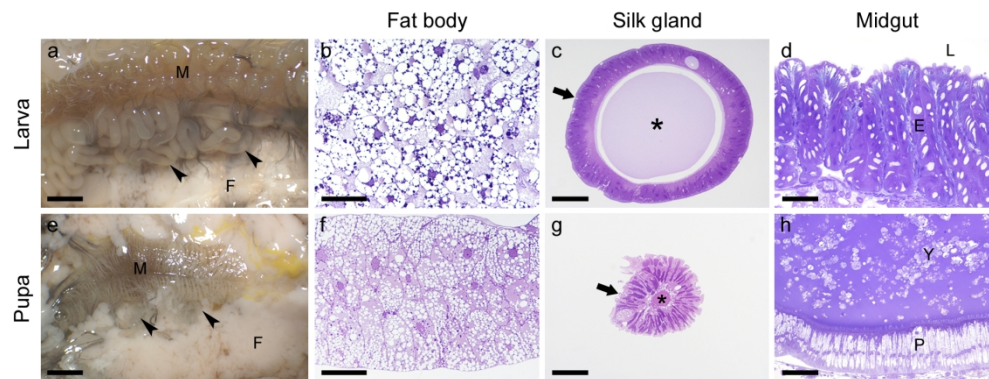
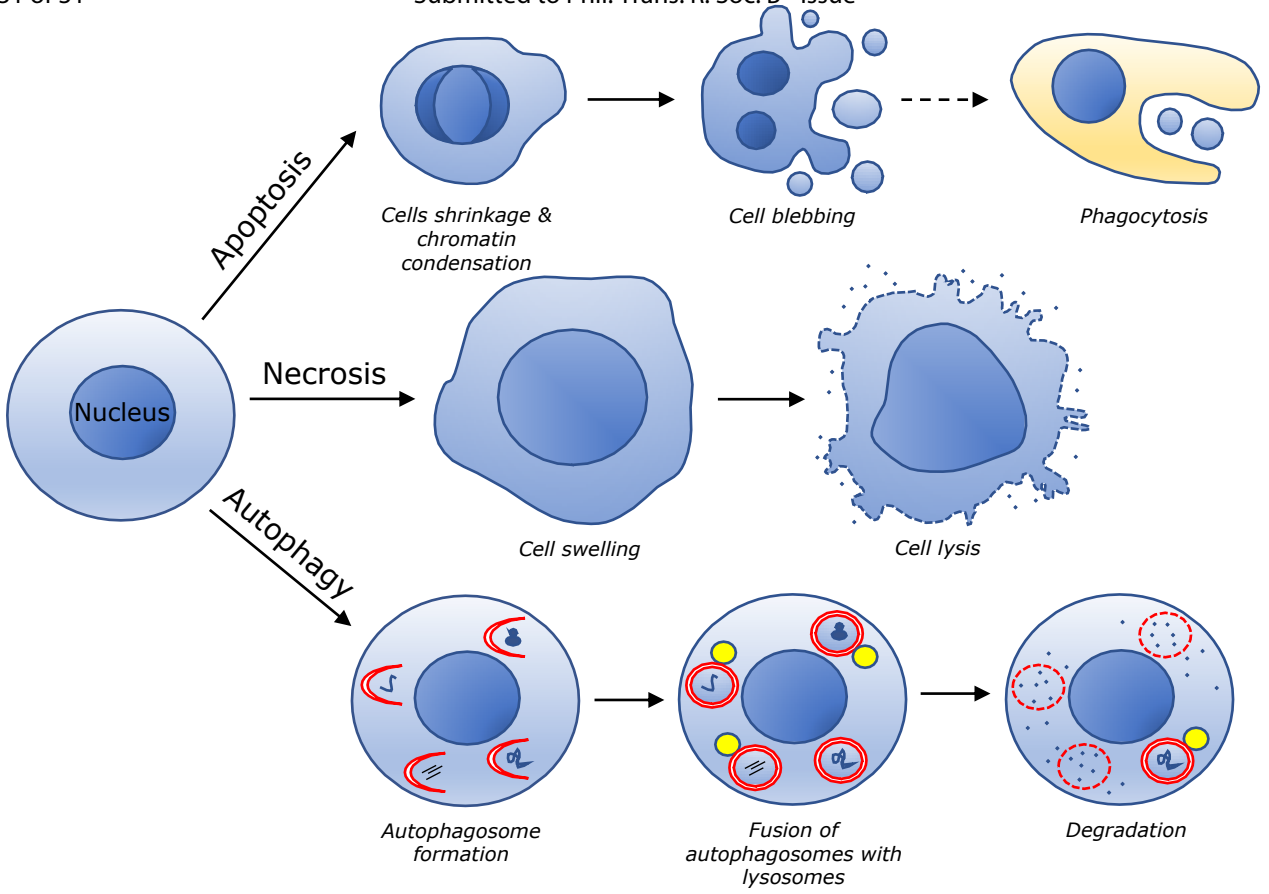


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Bars: 25 mm (a,e); 50 μm (b,f); 200 μm (c); 100 μm (d,g,h)

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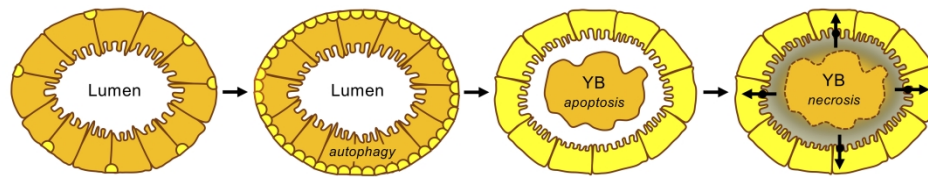


Figure 3. Schematic representation of the remodeling process that occurs in silkworm midgut during metamorphosis. Larval epithelium is indicated in orange, pupal-adult epithelium in yellow.

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