

Ontogeny of the pupal salivary, hypopharyngeal and mandibular glands and the
role of apoptosis during metamorphosis in *Apis mellifera*

by

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ABSTRACT

Amongst the most studied of the social insects, the honey bee has a prominent place due to its economic importance and influence on human societies. Honey bee colonies can have over 50,000 individuals, whose activities are coordinated by chemical signals called pheromones. Because these pheromones are secreted from various exocrine glands, the proper development and function of these glands is vital to colony dynamics. In this thesis, I present a study of the developmental ontogeny of the exocrine glands found in the head of the honey bee. In Chapter 2, I elucidate how the larval salivary gland transitions to an adult salivary gland through apoptosis and cell growth, differentiation and migration. I also explain the development of the hypopharyngeal and the mandibular gland using apoptotic markers and cytoskeletal markers like tubulin and actin. I explain the fundamental developmental plan for the formation of the glands and show that apoptosis plays an important role in the transformation toward an adult gland.

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Chapter 1: Introduction.

Honey bees (*Apis mellifera*) have been for a focus of scientific research for over 2300 years (Haldane 1955), but recent technologies and publication of the honey bee genome in 2006 (Consortium THGS 2006), permit a more detailed analysis of different aspects of the honey bee biology.

The honey bee is a holometabolous insect that undergoes complete metamorphosis, transitioning from its larval form to the adult, through intervening pupal stages. During this process, many internal organs will undergo changes, either losing functionality and going through dissolution, or gaining new capacities essential to adult survival. Other adult organs will develop from undifferentiated groups of cells called histoblasts (Snodgrass, 1956). Among the organs that will often undergo transformation are exocrine and endocrine glands, some of which are responsible for orchestrating the development into an adult, and others which have physiological functions that can change at different developmental stages. Endocrine glands directly secrete into the bloodstream a number of hormonal products that directly regulate the metamorphic processes.

Exocrine Glands

Exocrine glands have the ability to release their secretions to the exterior of the insect through a duct (Snodgrass, 1956). Exocrine glands can be classified into

pheromone producing and non-pheromone producing (like the salivary glands) structures. Pheromones are chemicals produced by an organism, that when released into the environment affect the behavior and physiology of members of the same species (e.g. sex or trail pheromones). Of the major exocrine glands found in honey bees, the mandibular glands are in the pheromone-producing category while the salivary and hypopharyngeal glands are categorized as non-pheromone producing.

The best studied exocrine glands in insects are the salivary glands. Three types of glandular cells have been identified according to the localization of these cells in relation to the insect cuticle and to the way the secretion is produced and discharged, based on ultrastructural data (Noirot and Quennedey 1969, 1974, 1991). Class 1 cells, are secretory epidermal cells and their secretions need to cross the cuticle to be released. This class is frequently associated with other cells (Blum, 1985). Class 2 cells are surrounded by epidermal cells and are not in contact with the cuticle. In this class, the secretion must pass through class 1 cells before crossing the cuticle via pore canals. Class 3 cells are isolated from the cuticle and possess a chitinous canal, which connects the glandular cell with the insect cuticle and drains the secretion outside the insect body. In addition to the classification at the cellular level, exocrine glands have been further classified into five different types based on presence or absence of reservoirs, defined as spaces within the epidermal cells that hold secretions of these glands (Fig. 1). The classes

are: epithelial cells with and without reservoir (Fig. 1A and B), secretory unit glands with and without reservoir (Fig. 1C and D) and glands (Fig. 1E) formed by the invagination of the intersegmental membrane (Billen, 2002).

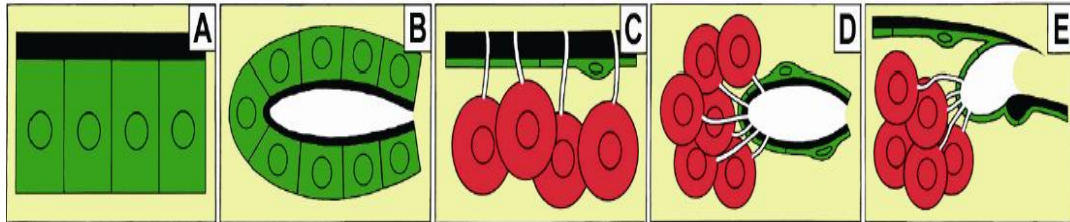


Figure 1. Classification of exocrine gland development based on the presence or absence of reservoir and the origin of the secretory cells. Image taken from Noirot and Quennedey, 1991

Salivary glands in larval honey bees:

The larval salivary gland is a long convoluted tube that runs from the spinneret in the mouthparts to the sixth abdominal segment where finally joins the end part of the larval gut. The initial function of the larval salivary gland is the production and release of brood pheromone, glycerol -1, 2-dioleate-3-palmitate (Koeniger, 1983). The methyl and ethyl esters making up the brood pheromone not only inhibit ovary development in workers but also have a primer effect that stimulates the hypopharyngeal gland development of nurse bees in young bees (Mohammedi et al., 1998). This pheromone manipulates the nurse bees to ensure the developing larvae get fed. The larval salivary gland also acts as a reservoir of fatty acid esters that constitutes brood food (food required by the honey bee larvae that is released by the hypopharyngeal glands) (Le Conte et al. 2006). Once the larvae reach the 5th instar, the salivary glands are used to make the silk needed for the pupal cocoon (Silva-Zacarin et al., 2007). At the beginning of the pupal period, the salivary gland nuclei disintegrate and cells become masses of irregular cytoplasm (Snodgrass, 1956).

Salivary glands in adult honey bees

During the transition to adulthood, the salivary glands undergo major changes in both form and function. The salivary glands in adult honey bees are also known as labial glands and are composed of two parts. The portions lying

posterior to the brain are called the post cerebral glands or cephalic glands and the portions in the thorax, just below the thoracic muscles are called the thoracic salivary glands. Both these glands use a common duct to open into the salivarium, a small pocket within the oral cavity that contains the opening of the salivary duct in an insect (Goodman, 2003). The thoracic and cephalic glands are reported to be larger in queens and workers. In drones (male bees) cephalic glands are seen to degenerate after sexual maturity (Poiani and Cruz-Landim, 2010). The cephalic and thoracic salivary glands differ in protein expression even though they have the same embryonic origin (Poiani and Cruz-Landim, 2010). Secretions of the thoracic portion, which is the largest exocrine gland in the honey bee, are aqueous and involved in sugar and honey digestion. The cephalic secretions are oily and used in the manipulation of honeycomb wax and lubrication of the mouthparts (Simpson, 1960, 1963, 1968).

My thesis project examines the different sequential developmental events that enables the larval salivary gland to metamorphose into adult glands. I also examine the morphological changes that the de-Novo hypopharyngeal gland undergoes with respect to the changes during metamorphosis and also cytoskeletal structures. Lastly, I also examine the changes in the cell thickness layer of a metamorphic mandibular gland and show the changes that the mandibular gland undergoes during metamorphosis. I used anatomical dissections, histology and immunohistochemical approaches to determine the

developmental events of the three glands. The salivary gland was studied during late larval (day 7-9), pre-pupal (day 10-11) and pupal stages (day 12-19) of the honey bee development. The hypopharyngeal and the mandibular glands were studied only during the pupal stages. The resulting morphological data that I collected will provide an important guide for future studies on the gene expression of these glands will aid in understanding of the general processes of development that may also guide the understanding of the differentiation of other structures during development and metamorphosis of different organisms.

Hypopharyngeal glands in Honey bees:

Hypopharyngeal glands are located in the bees head and is the source of brood food for the young ones in the colony. Two main groups of bees, the nurses and foragers are have very prominent hypopharyngeal glands. These glands are very plastic and vary in relation to the colony conditions (Maurizio, 1954; Moritz and Crailsheim, 1987). In foragers that have no young bees, these glands have been seen to redevelop (Huang and Robinson, 1996). The normal course of these glands are well known (Kratky, 1931, Maurizio, 1954, Free, 1961, Simpson et al., 1968, Moritz and Crailsheim, 1987, Suzuki, 1988, Crailsheim and Stolberg, 1989) but the development and maturation of these glands during metamorphosis is still unknown.

My thesis also tries to reveal the morphological and developmental changes that takes place in the gland during metamorphosis.

Mandibular Glands in Honey bees:

Mandibular glands are another very important exocrine gland in bees that are formed as an extreme reduction of a leg and converted into a feeding organ (Snodgrass 1956). These glands in workers extends upwards to the level of the antennal bases and even larger in queens but are really tiny in drones (Snodgrass, 1956). The mandibular glands have been involved in defense and alarm communications (Billen et al., 1998; Buschinger and Maschwitz, 1984, Hölldobler and Wilson, 1990), in others for nestmate recognition (Hernandez et al., 2002) and fungal growth inhibition (Marsaro Junior et al., 2001) and also serve as the source of sex pheromones ((Ayasse et al., 2001, Topoff and Greenberg, 1988). This thesis tries to show the changes in the developmental events in this gland by looking at the morphological changes during metamorphosis.

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Chapter 2:

Ontogeny of the pupal salivary, hypopharyngeal and mandibular glands and the role of apoptosis during metamorphosis of *Apis mellifera*

Abstract: The head exocrine glands in honey bees consists of the labial (salivary), hypopharyngeal and mandibular glands. All the three glands together form the salivary system in honey bees. Even though a few studies have examined the embryonic development of these glands, a detailed description of the changes that happen to these glands during metamorphosis was missing. Here, we investigate changes to the salivary glands during larval to adult maturation. Ontogeny of these glands was monitored using apoptotic markers (caspase 3), histology utilizing hematoxylin and eosin staining, cytoskeletal markers like tubulin and actin and dissections. During this experiment we were able to pinpoint the time of initiation of apoptosis in the larval salivary gland. This was found be as early as day 7 of the larval stage. We were also able to determine that the metamorphosis of the larval gland happens in a very sequential way, with the thoracic salivary glands developing from the remnants of the larval salivary glands, followed by the head salivary portions budding out of the thoracic portions, with differentiation and migration giving rise to the fully formed structure in the pupal stages. In case of the hypopharyngeal glands, a de-novo gland is seen to emerge after the larval stage is over and the first evidence of the

gland was visible in the late white pupal stage. This gland was seen to show signs of pruning and thinning of glandular components to give rise to a functional hypopharyngeal gland in an eclosed honey bee. The mandibular gland also showed apoptotic signatures in remodeling of the glandular components and showed signs of thinning too.

Keywords:

Exocrine glands, ontogeny, cytoskeletal structures, fluorophore, metamorphosis, honeybee, salivary gland, mandibular gland, hypopharyngeal gland, actin, tubulin, apoptosis

Introduction

The salivary glands of insects originate in the labial segment and are highly branched structures that secrete saliva within the oral cavity. Their exact form and function varies across species relative to their role in supporting feeding behavior. The glands are composed of clustered acini in honey bees (Arnold 1978; Kratky 1931; Poiani 2009; Schönitzer 1990), much as they are in locusts and cockroaches. In contrast, their structure is tubular in Lepidoptera, Siphonaptera, Heteroptera (Haridass 1981; Cobben 1978; Azevedo 2007; Ambrose 1999) and Diptera, and but highly complex in the hemiptera (Miles 1972, 1976; Hori 1969; Edwards 1961; Anhê 2008; Agnes 1990; Baptist 1941). For some species, including honey bees,

there is also considerable intra-specific variation in gland structure dependent on individual age and caste.

The salivary gland in honeybees become more active in laying queens while in the drones they degenerate as adults. The secretions of these glands vary depending on the age as well as the position of the glands depending on whether they are in the head or the thorax. (Poiani et al, 2010). The thoracic salivary, being the largest exocrine gland in the honey bee, produces a watery secretion with many digestive enzymes that are involved in honey and sugar digestion as well as pollen and wax moistening (Simpson 1960, 1963), whereas the cephalic salivary produces an oily substances utilized in wax manipulation and lubrication (Simpson et al. 1968). The cephalic glands are seen to increase in queens during egg laying activity (Katzav-Gozansky et al. 2001). The transformation of nectar to honey in a forager bee's gut is dependent on the secretion of the salivary gland with help of enzymes like invertase, glucose oxidase and amylase (Oddo et al. 1999; Maurizio, 1975; Doner 1977).

The larval salivary glands arise during embryonic development from parasegment 2 (PS2) of the ventral wall of the larva. They bilaterally traverse the entire length of the larval body from the anterior end to the posterior end, where they connect to the hindgut. Even at this stage, the glands are highly convoluted tubes that can be easily seen using any nuclear stain (Emmert, 1968; Snodgrass, 1956). This simple larval salivary gland then gives way to a highly branched

structure made up of two parts, the head salivary and the thoracic salivary gland (Snodgrass, 1956). The transition to this new morphology, primarily due to changes in the epithelial tissues, starts during pupal development, but a detailed description of the events that brings about the change is missing.

The remodeling of epithelial tissues using branching morphogenesis leads to the development of highly complex and ramified tubular networks in embryology.

This process has been extensively studied during development of vertebrate lung, kidney, vascular systems and numerous glands (Metzger et al, 1999, 2000) and also in *Drosophila* larval tracheal development (Krasnow, 2004). Apoptosis, or cell death, is an integral part of this process. Three types of apoptosis have been identified (Glucksmann 1951). First, is phylogenetic death, which occurs during the loss of vestigial organs. Second is metamorphic death, which occurs during the remodeling of organs during metamorphosis. Third is histogenic death, which occurs regularly during the differentiation and maturation of an organism. For the transition of honey bee salivary glands, the primary process appears to be metamorphic death. In *Drosophila*, this process is initiated by a pulse of 20-hydroxyecdysone (Ridderford 1993), resulting in the destruction of the salivary glands 15 hours later (Jiang et al. 1997). Dorstyn et al. 1999 established that a set of caspases called DRONC (*Drosophila* caspases) are responsible for triggering larval salivary gland apoptosis in fruit flies.

Reports of hypopharyngeal glands in bees and wasps goes back to Snodgrass 1956. The mandibular and pharyngeal glands have been classified as “Leydig Dermal gland” (Lukoschius 1962a). Weber 1954, mentions them as complex glands that develop from single gland elements and have single secretory ducts. Each stem cell of these glands, in *Formica partensis* generates five cells in four differential mitotic divisions (Lukoschius 1962a), a degenerating cell, the gland cell, the switch cell, the duct cell and an epithelial cell (Emmert 1964). In *Apis* however, the degenerative cell has been disregarded and only the gland cell, duct cells 1 and 2 and the epithelial cell is seen. Painter (1945) suggested that the each of the “gland cells” is apical and in contact with the “basal cell”.

These hypopharyngeal glands are Class III dermal glands in hymenopterans that are characterized by multicelled units in adult bees. They are comprised of 15-20 secretory cells each with its own a canal cell that is surrounded by cuticular microtubules. The cuticular microtubules are continuous with the end apparatus that is held together by actin rings. (Kheyri et al, 2012, 2013).

Mandibles are considered to be a result of extreme reduction of a leg that is converted into an organ of feeding (Snodgrass 1962). The coxa of the leg of the insect is believed to be retained with the elimination of the telopodite depending on the feeding habits of the insect (Snodgrass 1962). They are a pair of sac like

glands that lie just above the mandible in adults have been studied in relation to the pheromones they secrete and how these pheromones influence various aspects of the insect life (Smith and Roubik 1983, van Zweden et al 2011, Cruz-Lopez et al, 2005, 2007, Schorkopf 2009, Gary 1961). The worker mandibular gland is made up of a cuticular intima lined cavity that is elaborated by flat epithelium and numerous type 3 glandular units (Nedel, 1960). This gland has been in study since the early 1960's for the "queen substance" that has releaser and primer effects and also for an inhibitory pheromone (De-Hazan et al 1989a, Lensky and Slabezki 1981). Cell morphology of these glands (Abdalla and Cruz Landim, 2002), ultrastructure (Dezahan et al 1989), has been studied in details. Age related changes in the mandibular glands has also been seen in the drones (Lensky et al, 1985). The ontogeny of the mandibular glands has been studied in the ant *Formica parthensis* and *Apis mellifera* (Emmert 1968) but a detailed description of the changes happening in the different stages of metamorphosis was missing.

According to Emmert (1956) the primordia of the mandibular gland is already set apart early in pupal development and the cells can be divided into 3 different areas based on nuclear shapes, stringy cells (M, area 1) that differentiate into muscle fibers, layers with ellipsoidal nuclei in the middle (area 1 and 11) and the epidermal layer with small cells with spherical nuclei (area 111). The cells of the ventral sac wall consists of the area 1 cells and the dorsal area of the sac by

the area 11 and 111 cells. The gland primordia reaches its final destination by day 13 and finally the area 111 cells become the secretory cells. (Emmert 1956).

In order to determine if honey bee salivary, hypopharyngeal and mandibular glands undergo similar caspase activities to that of *Drosophila* during metamorphosis to the adult form, I used Caspase 3, one of the executioner caspases, and DAPI staining to determine where and when apoptotic cells occur in the larval salivary glands of honeybees. I also tracked morphological changes of the gland structures during the larval and pupal stages using dissections, histology and immunostaining.

Materials and Methods

Rearing of the honey bee larvae. Honey bees are holometabolous insects that undergo complete metamorphosis over successive stages of development from egg to larva to pupa and finally to an adult. (Fig. 2). Two queens of *Apis mellifera linguistica* were caged at the ASU Polytechnic Campus Bee Lab multiple times on different days to provide the age appropriate larvae and pupae needed for the experiment. The same queens were used to avoid genetic variability and were chosen for the rate at which they laid eggs. Each queen was caged using a 10x 8 inch queen excluder on an empty frame and left in the colony for a period of 12 hours for the queen to lay eggs. Afterwards, the queen was released and the excluder was put back in place to keep track of the eggs laid. This permitted

tracking of larval age, which was necessary observe development of the glands every 24 hours. Age appropriate larvae were scooped out of their individual cells and prepped according to the protocol for each of the staining methods.

Honey bee workers undergo a 21 day development period from egg to adult, first 3 days as an egg , the next 5 -6 days as a larva, then pre-pupa and four distinctive pupal stages before they eclose on day 21 (Snodgrass 1956). There are 4 distinctive eye color changes during pupal development that I used to categorize the age of the pupa during my experiments. The pupae is white eyed during day 13-14, pink eyed during day 15-16, brown eyed during 17-18 and finally maturing into a black eyed pupae during day 19-20 and the metamorphosed pupa eclose on day 21 into an adult bee. Maturation in the salivary, hypopharyngeal and mandibular glands were studied during metamorphosis under natural rearing conditions using a triple cohort experiment by caging the queen and age marking the pupa at the Bee lab at ASU East. The frames of brood were extracted from the hive the specific days and pupae were dissected under Ringer solution or PBS depending on the procedure used for their study.

Staining of larval salivary gland with DAPI. Twenty five larvae, 5 each from day 7, 8, 9, 10 and 11 post-hatch, were dissected in phosphate buffered saline (PBS; NaCl 128 mM, Na₂HPO₄ 16.7 mM, KH₂PO₄ 20 mM, pH 7.4) at room temperature. A pin was used to immobilize the larvae and an incision was made

on the dorsal side of the larvae. Buffer saline in a 3 cc syringe was used to spray the larval tissue intermittently to expose the glands after removing the overlying tissues. Both of the salivary glands were then carefully extracted using a tweezer and placed in a 9 well plate with more saline buffer.

Different trials with varying timings were performed to determine the best staining protocol for the whole glands. The best protocol appeared to be exposure of the glands to 60 min of freshly prepared 4% paraformaldehyde (PFA), followed by three washes in PBS again for 10 min each. Later on the tissues were transferred to a combination of DAPI (4'6-diamidino-2-phenylindole) and PFA in the ratio of 1.5 μ l: 3000 μ l; DAPI: PFA and left in a rocker for 30 min. The samples were washed again with PBS three times for 10 min each. The glands were then mounted on a slide using Flouragel (Fisher Scientific, Hampton, NH) and observed under epi-fluorescence microscope (Olympus BX53 Upright Epifluorescent Microscope with a Hamamatsu ORCA-03G high resolution CCD camera and a Prior Scientific Optiscan ES103 XYZ motorized scanning stage for automated tiling)

Immunohistochemistry using Caspase3: Caspase 3 was chosen for the assays, as it is one of the executioner caspases and is downstream of all the different proteins that are needed to release Cytochrome C that cleaves DNA. A positive control of a glutamatergic neurons in five day old mouse cortex was used for caspase 3 activity (Fig. 3A, B). For a negative control, day 4 larval glands were used as apoptosis was not expected that early in development.

Three replicates of dorsally slit whole larvae for each age cohort (9, 10 and 11 days) were preserved in 4% PFA overnight, for immunohistochemical processing. These samples were then transferred to 30% sucrose for 30 min and 10 μ m sections were obtained using a cryostat (Leica CM1950 Cryostat). The sections were placed on slides and then transferred to a slide warmer for approximately 20 min. Slides were washed with 1% PBS three times for 10 min each. After that, they were treated with Anti-Rabbit Caspase 3 primary antibody (1 μ l:1000ul in 3% PBST) and normal docking serum (NDS) (1 μ l:1000ul) and incubated overnight at 4°C. This was followed by five 10 min washes with 1% PBS; then the tissues were incubated with Donkey anti-rabbit secondary fluorophore 567 (Thermo Fisher; 2:1000) and DAPI 405(1:1000) with NDS for 2-3 hours. The slides were then washed in PBS for five times and mounted in Flouragel. Imaging was done using an epi-fluorescent microscope and further processed using Adobe Photoshop.

Histology for salivary, hypopharyngeal and mandibular glands: Three replicates of age-marked dorsally-slit larvae of days 9, 10 and 11 and pupae (head and thorax) of day 13-14 (white eye), day 15-16 (pink eye), day 17-18 (brown eye) and day 19-20 (black eye), were preserved in 4% PFA overnight and dehydrated the next day, using an ethanol series (30%, 50%, 70%, 80%, 95%, 100%) diluted with ultrapure water at room temperature. The samples were left in each ethanol concentration for 45 min, and then were placed twice in 100% xylene for 30 mins

each, then once in 1:1 xylene: paraplast for 30 min, and then twice again in 100% molted paraplast for an hour each. This was done to maximize the penetration of the paraplast and remove any remnants of ethanol. The larvae and pupae were then embedded in paraplast in plastic molds. The next day, the blocks with the samples were cut with a microtome (American Optical Spencer Model 820 Rotary Microtome) to produce 9 μm sections. These sections were then stained first with hematoxylin to color the nucleic acids in the cells blue and then with eosin to color the protein components of the cell pink. This was done to enhance differentiation between cellular and non-cellular components in the histological sections. These sections were then screened using a compound microscope to determine the presence or absence of glandular structures.

Anatomy study of the glands using dissections: Live pupae of the four age groups were immobilized in insect saline using a pinning needle through the thorax, then dissected. An incision was made on the vertex of the head and cut around the eye sockets to expose the salivary glands in the head. Another longitudinal incision was made on the thorax just below the vertex, cutting posteriorly towards the abdomen to expose the thorax interior. Constant use of saline water in a syringe helped displace unwanted structures to reveal the salivary glands, mandibular and the hypopharyngeal glands underneath. These glands were then stained with methylene blue with 1% PBS solution for a few minutes and then removed from the pupal carcass and placed in a slide to study

the anatomical changes that happen during the different stages of development. Pictures were taken using a compound microscope and was then compared with the histological sections of the pupae.

Measuring the cell thickness of the mandibular gland cell layer during pupal development: Using histology with H and E staining, the average diameter of the cell layers of different pupal stages were measured using the ocular micrometer in a compound microscope. The real measurement of the cell layers was a little underestimated because of the process of histology.

Immunocytochemistry of Hypopharyngeal glands using tubulin and actin: 3 of the age marked pupae from the same age cohorts were dissected for their hypopharyngeal glands. Incisions were made in the top of the head (vertex) using a micro scissor, and cut along the boundary of the compound eyes along the lateral margins of the frons. After removing the frons, the hypopharyngeal glands were carefully removed from the head capsule and preserved in freshly prepared 4% PFA overnight. All dissections were done under a dissecting microscope using phosphate buffer saline (NaCl 128 mM, Na₂HPO₄ 16.7 mM, KH₂PO₄ 20 mM, pH 7.4).

1. Agarose sections: Different stages of the hypopharyngeal glands were treated subsequently with primary mouse anti beta-tubulin (1:2000; MAB3408, Chemicon, Merck Millipore) in combination with an Alexa Flour 488 conjugated

goat anti mouse antibody (1:400; A-11001, Molecular probes Eugene) to label tubulin rich fibers.

The glands were also double labelled with Phalloidin (647) to visualize the actin filaments in the developing glands. The fixed glands were washed with PBS 5 times for 5 min each before embedding them in 8% agarose(Agarose11, no 210-815, Amresco, Solon, OH)and sectioned at 70 um using a vibratome(Leica VT1000S). After staining was done the sections were mounted in slides using Flouragel(Bio media, cat. no. 17985-41) and imaged using laser scanning confocal microscope(Leica TCS SP5 AOBS, Leica Microsystems AG, Wetzler, Germany) equipped with argon, Krypton and three diode lasers. Three HC PI APO objective lenses (20 X/0.7NA imm) were used for image acquisition. The glands were scanned under the individual channels and further processed using Adobe Photoshop.

2. Whole mounts: 3 whole mounts of the hypopharyngeal glands were also performed with primary mouse anti beta-tubulin (1:2000; MAB3408, Chemicon, Merck Millipore) in combination with an Alexa Flour 647 conjugated goat anti mouse antibody (1:400; A-11001, Molecular Probes Eugene) and Phalloidin (560) to visualize the changes in the microtubules and actin filaments in the developing glands. The protocol used was the recently established one. (Groh et al.) The staining was followed by an ascending series of ethanol dehydration before it was mounted in methyl salicylate(M-2047, Sigma Aldrich) The whole

mounts were then imaged using laser scanning confocal microscope(Leica TCS SP5 AOBS, Leica Microsystems AG, Wetzlar, Germany) equipped with argon, Krypton and three diode lasers. Three HC PL APO objective lenses (20 X/0.7NA imm) were used for image acquisition. The glands were scanned as individual channels and further processes using Adobe Photoshop.

Results

The larval salivary glands: The honey bee larval salivary gland is made of two parts (Fig. 3; Emmert 1968). The anterior portion, which has been described as the export system, includes the unpaired export duct (UAG) and the paired export part (PAA) which is relatively longer than the UAG (Emmert 1968). The posterior portion, separated from the anterior portion by a constriction (C1), is referred to as the winding gland tube (Emmert 1968). It has three distinct zones: the anterior (AZ), middle (MZ) and posterior (PZ). The posterior portion is also called the gland hose (Emmert 1968). We found that the export system, has a second constriction (C2) posterior to the export system (Figure 3B-D). This includes the region described as the front cells or Anterior Zone (AZ) (Emmert 1968). The C2 is seen to separate the middle portion of the winding gland tube, Middle Zone (MZ) and the gland hose, Posterior Zone (PZ) from the anterior portions of the winding part of the larval gland. This constriction was not described previously and its function

is still unknown. This constriction (C2) very clearly demarcates the export system from the MZ part of the winding region in the honey bee larval salivary gland.

Cell density appears to be dramatically reduced between C1 and C2 compared to the winding portion of the larval gland. Very little staining with methylene blue was observed between C1 and C2, indicating the possible absence of cellular components (Fig 4F). In contrast, the winding regions showed compact cells with very conspicuous nuclei with several nucleoli within them (Fig 4E). The number of nuclei varied from two to six per cell (Fig 4E) throughout the length of the winding part PZ (Fig 4E)

Beginning of Apoptosis in Larval Salivary Glands

Whole mounts with DAPI. Whole mounts of the different age cohorts of the larval salivary gland using DAPI (Alexa 405) to detect apoptotic cells, did not yield confirmatory results. Some apoptotic cells were seen in the whole mounts but this may have been due to damage incurred during the staining process. Therefore, DAPI staining alone was not sufficient for detecting apoptotic activity. To better understand the apoptotic phenomenon, we used Caspase3 along with DAPI to look at confirmatory signatures for apoptosis.

Staining with Caspase. All the cryosections of the day 7-11 to 11 larvae and the prepupal stages were screened for caspase activity to determine the onset and location of apoptosis in the larval glands. Very mild caspase activity was seen as early as day 7+ 24 hours (Fig. 4B) which might indicate that the first pulse

of 20 hydroxyecdysone is secreted around day 7 of larval salivary development. This is a couple of days before cell capping on day 9, which typically marks the beginning of salivary gland transition. The apoptotic cells were restricted to the posterior portion of the salivary gland, behind the AZ portion of the winding part. Day 8 larval salivary glands showed an increase in caspase activity compared with day 7 larvae. We observed this regular increase in apoptotic cells till day 11, by counting the number of apoptotic cells seen for different larval glands under the same magnification. (Fig.5 and 6).

Larval salivary gland transformation during pre-pupal and early pupal stages .The larval salivary glands in honey bees start out as a bilateral tubes with distinct regions (Emmert 1968). The larval gland is proposed to cleave with the help of apoptosis around the posterior end of PAA, just before the AZ from the findings of the caspase assays that were performed (Fig 5 and 6). The remainder of the larval gland forms growth zone, or anlagen TA (Fig 8, day 12±12 hours) for the formation of the thoracic gland and HA for the head salivary (Fig 8, Day12±12 hours). After the winding portion of the gland disintegrates, new growth zones (Fig. 8, HB and TB) become evident around day 12 ± 24 hours and this growth zone becomes the thoracic bud (TB). The next 12 to 24 hours are associated with splitting of the bud into separate branches. These branches were seen to develop into the primary and secondary branches of the thoracic salivary gland that finally gives rise to future tubular acini that are the secretory units of the glands (Cruz

Landim 1967, Emmert 1968). The portion of the paired exit duct (PAA) anterior to the thoracic bud (TB) is proposed to undergo elongation and expansion to give rise to the thoracic reservoir (R) by day 14 (Fig. 9 D), but needs more marker specific experiment's for confirmation.

During day 12 \pm 12 hours, another anlagen (Fig 7, HA) is seen at the junction of the UAG that splits to form the PAA sections. This anlagen gives rise to the head bud (HB) that extends by continuous cell division during the next 24 hours. The developing head bud in another 24-48 hours develops two lateral side buds that ultimately give rise to the two lateral cephalic branches of the head salivary gland (Fig. 8)

Development of thoracic salivary gland in late pupa. Starting on day 13, the pre-pupal thoracic bud undergoes proliferation of cells (Fig. 9, 10). This initiates branch formation. The thoracic salivary reservoir (R) (Fig. 9B) develops from the paired exit duct portion of the larval salivary by enlargement and cell elongation. The primary branches develop secondary branches (SB) and tertiary branches (TB) by day 17. The tips of the branches then differentiate into tubular secretory units called alveoli (A) (Fig. 9, 10). This was well documented in both the histology and the dissections.

This process provides evidence of organ-specific terminal differentiation. These tubular units penetrate through and extend posteriorly and ventrally beneath the thoracic muscles. Then they infiltrate the thoracic regions below the

digestive canal but above the nerve cord. The paired thoracic salivary glands are complete at day 20 of metamorphosis (Snodgrass 1956). The adult glands have a reservoir with many tubular secretory units. Each of these secretory units have ducts that connect to the reservoir that makes it a fully functional adult thoracic salivary (Snodgrass 1956).

Development of the cephalic salivary gland in late pupa. The development of the thoracic salivary gland is accompanied by the evagination and development of the cephalic salivary gland on day 12. After the head bud (HB) is formed from the anlagen between the UAG and PAA (Fig 9, HB), the bud migrates from the thorax towards the posterior portions of the cranium through the occipital foramen (Fig. 7, 11, 12) by day 13 (Cruz-Landim 1967). The primary branches for the head glands get laid down by day 14 and some evidence of secretory unit formation is seen (Fig. 12B). These primary branches continue to extend laterally and get interspersed with the hypopharyngeal gland and mandibular muscles of the head (Snodgrass 1956). By the late pink eyed stage (Fig. 12C), we see secondary and tertiary branching followed by an increase in the appearance of the secretory acini (Fig. 12C, D). These branching events continue well into day 17 of metamorphosis (Fig. 13A-C). The secretory units, at the tip of these branches, are alveolar in nature. Most of the secretory cell surfaces are non-sclerotized and trachea are seen to penetrate these secretory units by day 19 of metamorphosis.

Histology of the salivary gland development: Histology of the different stages of pupal salivary development shows the same stages seen during dissection. Fig 14A represents the stage seen in Fig 7A. This is the stage after the partial apoptosis of the larval salivary. This is the stage when the anlage are formed and it paves the way for the development of the head and thoracic salivary. Fig 14B is representative of the primary branch formation of the head salivary seen during the pink eyed pupa. We don't see any acini formation during that pupal period. Fig 14 C is the representation of the thoracic bud (TB) seen during dissection of the salivary gland during the early white eyed pupa stage (Fig 7B). This bud, in the next few hours develops into the thoracic reservoir and branches of the thoracic salivary. Fig 14D is part of the head salivary during the brown eyed pupa that shows acini formation and the presence of the reservoir within the acini. Fig E and F confirms the formation of the thoracic reservoir seen during dissection (Fig 10A) and the bilateral branches that makes up the matured thoracic salivary gland (Fig 10B).

Hypopharyngeal gland results:

Hypopharyngeal glands development during metamorphosis: According to Emmert 1964, the hypopharyngeal glands in honey bees are made up of epithelial cells made up of mid-sized nuclei(EZ), a small sized nuclei in the exit area of the secretory duct(KZ), mid-size nuclei in basal area of the ducts (SZ) ,

cells with strongly vacuolated cytoplasm that develops into gland cells(DZ) and some basophilic cells , with evenly granulated cytoplasm(BZ).

During metamorphosis, these hypopharyngeal glands of day 13-14 pupa shows very little clustering of cells. The cells are more or less concentrated near the central duct and the thickness of the duct is relatively uniform. Histology of this stage shows a very clear central canal made up of mid-sized nuclei (EZ) (Fig 15). The secretory duct (KZ) and basal area of the ducts (SZ) are not very clear and some sort of clustering of cells that may include the other two types of cells (DZ and BZ) mentioned by Emmert. The individual cells shows multiple nucleoli (Fig 15 A). Day 15-16 of the pupa shows a lot clearer central duct system as the surrounding cells are seen to be extended outwards, away from the central canal with the help of extending microtubules from the central duct (Fig 15 C). At this point we see a small sized nuclei in the exit area of the secretory duct(KZ), mid-size nuclei in basal area of the ducts (SZ) also called duct cell II (Emmert 1962) that gives rise to the canal cells composed by microtubules, along with the EZ, DZ and BZ cells. This stage also shows the extension of the KZ and maybe SZ cells facilitating this movement of the DZ and BZ cells away from the central duct (Fig 15 E). We also see that the tube shows inconsistency in cell density. At this point, the density of the cells around the duct also seems to decrease which might suggest the role of apoptosis in the maturation process. Day 17-18 of metamorphosis shows distinct secretory cells (DZ and BZ), together with

extension of the microtubules and continued migration of the secretory units away from the central duct. This can be determined by measuring the length of the microtubules during different stages. The secretory units are not very clearly distinguishable from one another and the cells show the presence of large nuclei inside the secretory cells that can be seen with the hematoxylin and methylene blue stains. The clusters start to look more like the matured acini's at this point. This is followed by day 19-20 day old hypopharyngeal glands where the cuticular microtubules inside the secretory units can be clearly seen with large nuclei. At this stage we also see the compaction of the acini, maybe due to increase in cell adhesion. Each cell in the acini are connected to the central duct with its individual collecting duct made of microtubules and the canal cell, which is unlike what is seen in the salivary and mandibular glands.

Role of Apoptosis in the development of the Hypopharyngeal gland:

Apoptosis in the above mentioned stages of hypopharyngeal glands were also studied using Caspase 3 antibodies. Whole mounts using the similar protocol used for salivary glands were done and the glands were counter stained with Donkey, anti-rabbit secondary fluorophore, 567(2:1000) and DAPI 405(1:1000) with NDS for a couple of hours after which the slides were washed in PBS for 5 more times and mounted in Flouragel (Bio media, cat. no. 17985-41). Imaging was done with an epi-fluorescent microscopy.

The day 13 -14 cross-section of the gland shows slight activity of Caspase 3 around the central duct. This might facilitate the movement of the canal cell away from the central duct leading to migration of the developing cells (Fig 16, 17, 18). Day 15- 16 pupal stage shows progressive increase in apoptotic activity around the acini units suggesting the possibility of cell transformation, induction, remodeling of the cells that will form the secretory cells, the canal cells and well as the microtubules. In the later stages, caspase 3 activity is seen mainly around the individual cells of the secretory units and finally around the interior of the acini which might be related to cell maturation, changes in the microtubule and actin cytoskeletal structures that is associated with maturation of the glands. They could also be indicative of end of proliferation of the microtubules (canal cells) (Fig 17, 18).

Tubulin and actin changes in the Hypopharyngeal glands: The different stages of the hypopharyngeal glands were stained with anti- beta-tubulin and actin to look at the cytoskeletal structures and how they changes during all these developmental stages during honey bee pupal developments. Both whole mounts and agarose sections were done. For whole mounts, the protocol was the same used for caspase3 and for the agarose sections the sections were cut at 70 u using a vibratome and these sections were imaged using laser scanning confocal microscope(Leica TCS SP5 AOBS, Leica Microsystems AG, Wetzlar, Germany) equipped with argon, Krypton and three diode lasers. Three HC PI APO objective

lenses (20 X/0.7NA imm) were used for image acquisition. The images were then further processed using Adobe Photoshop and Adobe Illustrator.

The cross section through the hypopharyngeal gland of the 13-14 day old pupa (Cross-section) shows microtubule tracks radiating out from the central duct along with some cells with big nuclei. This was not evident in the histology sections. The anti-beta tubulin staining shows that these microtubule tracts are concentrated in certain areas of the gland that in future might develop into the individual part of the canal cell that gives rise to the individual excretory ducts. These ducts emerge out of the DZ cells in the secretory acini of the matured gland. Two or three layers of cells are seen surrounding the central canal. These cells could be the progenitors of the secretory DZ or BZ cells in the future. The section also shows some smaller cells that could be proliferating cells. Each surrounding cell has a big nucleus that might suggest heterogeneity of cell types. Day 15-16 hypopharyngeal glands (transverse sections) (Fig 16) shows the central canal and the microtubules starting to disperse away from the central canal. This is similar to what is seen in histology with the microtubule tracts becoming longer. Actin rings are seen more clearly at this stage and are seen to be concentrated more inside the secretory cells. The Phalloidin stain seems to be outlining the secretory and the canal cells, similar to the findings of CellMask Orange (Richter et. al 2016). Stage 17-18 of the pupal hypopharyngeal shows a continuous trend of elongation of the microtubules away from the central duct and

the connection of the microtubules from the central duct to the acini becomes more obvious (Fig 16, 17). This stage is also marked with appearance of the actin rings around the nucleus, but it is still not clear where exactly the actin rings lie in each secretory cell. The microtubules are seen to separate out of the bundle at the entry site of each of the secretory cells and enter individual cells in the acini. Day 19-20, shows very clear actin rings outside the nucleus and we see the concentric rings going around the canal cell. The actin rings look like concentric rings that lie outside the nuclei and around the canal cell (Richter 2016), but the interconnections mentioned in the paper was not seen in 19-20 day old pupa.

Mandibular Gland results:

Apoptosis in mandibular glands: All the different cell types as mentioned by Emmert (1956) was seen in mandibular glands of the day 13 -14 pupae. The reservoir is fully formed and the multiple layers of cells showing distinct gland cell (DZ) are also evident in the white eye pupa. We also see other smaller cells that line the epithelia of the reservoir which are probably the EZ cells. As the metamorphosis progresses (day 15-16), the thickness of the DZ cells are seen to decrease around the reservoir (Fig: 19 A, B, C, D). This might suggest some apoptotic activity of the cells leading to reorganization of some cells. This trend is seen to continue in the other two stages (day 17-18 and day 19-20) of the gland and finally we see very prominent secretory cells (DZ) with a large nuclei (Fig: 19 D). In the last stage of metamorphosis, several nucleolus are seen inside the

nucleus that might suggest increase in rRNA production leading to preparation of secretory activity of the gland on maturation. There needs to be additional work done on when does these glands first show secretions.

Thickness of the mandibular layer: The thickness of the mandibular gland boundary layer during different stages of pupal transition was measured using histological sections stained with hematoxylin and eosin. The thickness was seen to be the maximum in 13-14 day old pupae and gradually decrease throughout the advancement of different pupal stages from day 13 to black eye (day 19-20) pupa. More distinct and defined secretory cells (area 3) were seen in the mandibular glands during the latter part of the pupal development. (Fig 19). There was significant difference in the thickness of the layers between day 13-14 and Day 19-20 ($p < 0.0005$), difference between day 15-16 and day 19-20 ($P < 0.0005$) and also between day 17-18 and day 19-20 ($P < 0.0005$)

Discussion

The main aim of the study was to extend the information provided by Emmert (1968) in the light of some modern technology and also to describe in details the changes happening to the head glands during the pupal period. This study revealed the detailed morphological events that take place during metamorphosis of the salivary gland from the larval to adult honey bee.

The larval gland was seen to show signs of apoptosis as early as day 7 ± 24 hours using DAPI and CAS3 staining. Cas3 was used in the study as it is one of the

downstream caspases that ultimately leads to disintegration of the DNA. Studies in *Drosophila* salivary glands have indicated the involvement of 20 hydroxyecdysone in the mechanism of transition of glands from larval to adult glands by apoptosis and that the first pulse happens about 12-15 hours before apoptosis (Ridderford 1993, Jiang et al. 1997). The indication of initiation of apoptosis as early as day 7±24 hours of larval salivary, suggests that the first pulse of 20 HE might occur as early as day 6±24 hours. This finding is important because even though studies have shown molting and metamorphosis is initiated by a pulse of 20 HE, the exact timing of the first pulse was not known. Once initiated, apoptosis occurred mainly in the region posterior to constriction1 (C1). From the dissections using methylene blue and evidences through Cas3 and DAPI staining, it is confirmed that the larval gland cleaves near C1 and the anlagen for the adult head and the thoracic salivary starts to show cell growth that leads to the development of the next stage of the glands. The larval salivary gland attain their maximum growth just as the larva is ready to spin its cocoon (day 9) after which they start to rapidly degenerate (Anglas 1901). From the apoptosis assays, we now know that this degenerative process starts as soon as day 7 and continues throughout day 10 of the pre-pupal stage. Contradicting Oertel's (1930) assertion that all trace of the larval salivary is gone by 70 hours after sealing of the cells, we now see that part of the larval gland is retained and finally reconstructed by the process of branching morphogenesis to give rise to a completely new and highly branched system.

In addition to regulatory control by 20 HE, the process of gland restructuring might also be influenced by changes in juvenile hormone (JH) titers that are seen during insect metamorphosis. Tian et. al 2013 and Romanelli et al 2016 also showed that 20HE titer in the hemocoel of the larva in the beginning of metamorphosis is responsible for autophagy. JH titers from the corpora allata have been seen to delay the process of metamorphosis in insects. Studies on mandibular glands in honey bees shows that the presence of the JH titers are also responsible for initiation of metamorphosis (Salles 2004, Fluri et al. 1982). JH has also been seen to effect DNA synthesis and apoptosis in caste specific differentiation of larval honey bee ovary (Capella, 1998). This initiation of apoptosis, seen as early as day 6, could also indicate the JH titer initiating metamorphosis in larval salivary glands in honey bees too. This requires more investigation about whether it is 20HE or JH that leads to the apoptotic cells we see by the end of day 6 in the larval salivary gland of the honey bee.

Another important finding was the observation of a second constriction that had not been previously described. This region might serve as a point of differentiation in the larval gland that might have functional significance during the larval growth. Since the region between the C1 and C2 showed more membrane reinforcement with actin-like rings (Figure 4F) and relatively few cellular components, it may be used as a structural support for the gland. In contrast, the MV and PV show high cell density with multiple nucleoli, suggesting

that this might be the synthetic region for pheromones, including the brood pheromones used to manipulate conspecifics (Conte et al. 2006). Also, by the end of the 5th instar larva (developmental stages between each molt), silk production is seen in the larval salivary gland. The multinucleated cells could also be a source of the silk fibrils (Silva-Zacarin et al, 2003, 2007).

The observed development and bilateral branching pattern of the head and thoracic salivary and also the presence of the ring like markings similar to those observed in the tracheal system (Krasnow, 2002) suggests that they too might be composed of structural proteins like tubulin and actin (Fig. 10D). This might indicate that patterning events of both these systems might be controlled by the same genes and transcription factors that regulate the development of the tracheal system in *Drosophila* and also some vertebrate organs. This might indicate common ancestry and common gene regulatory network across different phyla of animals. The involvement of Breathless, Branchless, Stumps, Ribbon and Anaconda genes (Kornberg and Krasnow 2000, 2003) and the role they play in the different phases of the salivary gland formation will also be very interesting to investigate.

While observing the different time points and events of the thoracic and head salivary development of the bee during metamorphosis, I could relate to some similarities to the branching morphogenesis process described in vertebrates and *drosophila* tracheal system (Ghabrial 2003). The genes and

transcription factors (TF) mentioned in *Drosophila* tracheal development, like the Breathless FGF factor and E26 transformation specific Transcription factor9 (ETS) (Krasnow 1996), may also play a role in determining branch formation in the salivary system in bees. The formation of anlagen, is seen in the case of thoracic salivary gland (Fig. 6B) on day 12 ± 12 hours and for the head development at day 12 ± 24 hours (Fig. 8C). This process is followed by the primary bud formation seen in both the head and thoracic salivary (Fig. 8C and 8D) with the formation of the head bud and the thoracic buds. This might be a very precise timing to see the involvement of Branchless FGF signaling (Krasnow, 2000) in the role of formation of primary branches. These buds then undergo a branching event that involves lateral cell elongation and migration of cells giving rise to primary branches (Fig. 8F). The acini or the secretory units of the thoracic glands are tubular in the thorax and alveolar in the head. This might suggest the presence of different gene expressions controlling the shape and secretions of two spatially different glands but of the same origin.

The process of development of the hypopharyngeal and mandibular glands in the bees is dissimilar from the salivary gland developmental pattern even though they are exocrine glands. The hypopharyngeal gland, unlike the salivary, is not found in the larvae but develops de- Novo during day 12 ± 24 day of pupal development (unpublished data) from an epithelial invagination near the lateral regions of the mandible. After initial differentiation, the pupal

hypopharyngeal gland undergoes thinning and pruning of cells during eclosion to give rise to the functional gland (unpublished data). The mandibular gland on the other hand does not show any primary duct formation during maturation, and is instead shaped primarily by apoptosis of peripheral cells (unpublished data). These different patterns of development suggest the work of alternative regulatory genes and probable transcriptional factors.

The head part of the salivary differs from the thoracic part of the salivary glands not only in the structure of the secretory units but also in the formation and position of the reservoir. The head salivary acini are Class-1, epithelial type (B) glands that have a reservoir (Fig. 1, chapter 1). The cells in the acini are arranged around the reservoir and each acini has the same arrangement. Ontogeny of these glands shows an epithelial origin (Lommelen et al 2003, Billen 2009). The thoracic salivary glands, on the other hand, show development similar to epithelial cell types D and E. The tubular glands have a central duct (Cd) (Fig. 10C) with secretory cells (Sc) arranged around it. All these tubular glands empty their contents into the reservoir anterior to the glandular units which then connect to the common duct that leads into the cephalic salivary gland. These developmental differences in the two sections might explain the differences in the secretory nature of these two glands. Through proteomic analysis of these two separate glands (Fujita et al 2010), it was found that the enzymes aldolase and acetyl CoA acyltransferase 2 are expressed more in the head salivary than the

thoracic salivary. In addition to that, imaginal disc growth factor (IDGF 4), which is a major component of royal jelly that may be involved in the growth and physiological changes in the bee's later life, is also produced at higher concentration in the head salivary gland.

The changes in the maturation of the hypopharyngeal glands during pupal development and its association with the changes in the morphological markers like actin and tubulin and also the role of apoptosis shows carefully controlled genetic regulation that needs further investigation. The developmental processes like cell migration, differentiation, determination also seems to play a very critical role in the maturation of the pupal hypopharyngeal to a functional adult hypopharyngeal gland with maturation of the secretory cells (DZ)

In summary, the results of this study make a significant contribution to understanding the changes that occur during the pupal phase in honey bee development the head exocrine glands in honey bees which play several key roles in the function of honey bee colonies. This study might also help in understanding the physiological roles that these gland plays in adult honey bee workers, queens and drones. By understanding the regulatory genes that control the proper development of these glands, we may be able to determine genes associated with vertebrate salivary glands development and disease.

My data demonstrates that i) apoptosis of the larval salivary gland occurs at C1 and as early as day 6 could be when the 20 HE is released for the apoptosis

to start ion day 7 of larval development. ii) the presence of C2 or constriction 2 in the larval gland also helped add to Emmert’s finding about different zones in the larval salivary gland. iii) the head and the thoracic salivary starts from anlage in the remaining salivary gland after apoptosis and shows very specific time changes that I was able to document. iv) using cytoskeletal markers actin and tubulin, I was able to see the changes in the hypopharyngeal glands during metamorphosis and also document the changes in the different kinds of cells mentioned by Emmert as the gland matures throughout pupation. v) The pupal mandibular gland also undergoes rearrangement of cells and maturation that was seen in the change in width of the glands.

Figures:

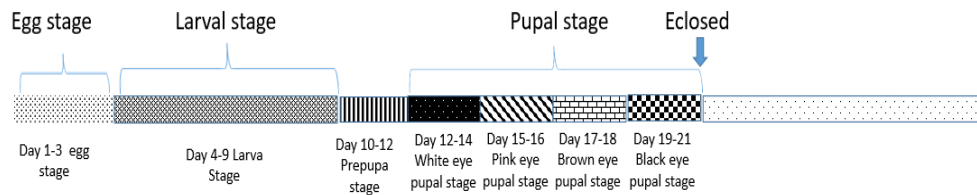


Figure 2: **Experimental timeline:** Overview of the experimental timeline of the honey bee life cycle. Double brackets indicate the time of the different stages of honey bee lifecycle from egg, larva, prepupa, pupa and adult. Arrowhead indicates the time point of eclosion.

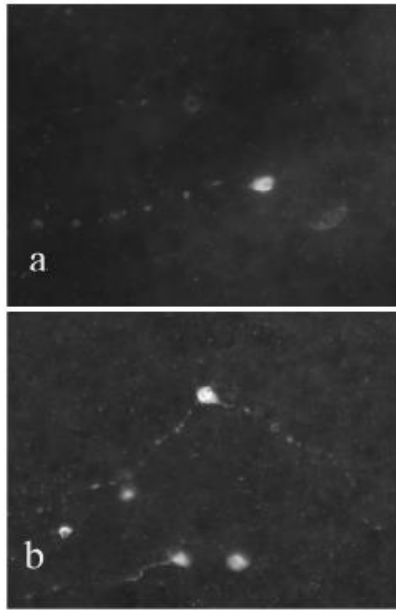


Figure 3. Positive control for Caspase3. A: glutamatergic neurons of a 5 day old mutant mouse. (20 μ m). B: 5 day old mutant mouse cortex showing the cell body and axon (20 μ m)

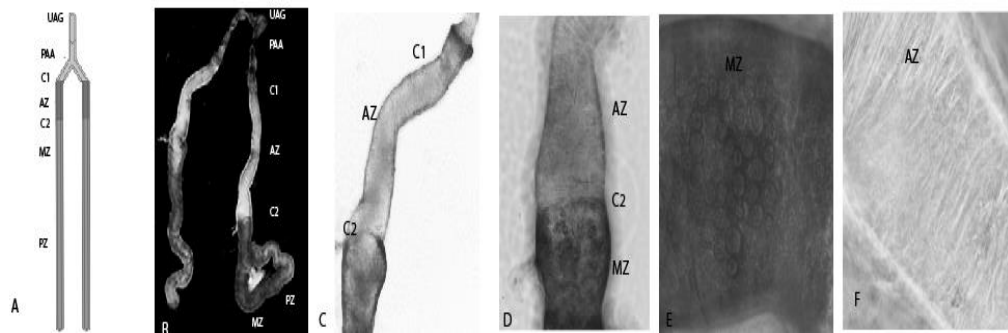


Figure 4: 8 day larval salivary gland in *Apis mellifera*. A: Different parts of the larval gland as described by Emmert (1968) (200µm), B: Evidence through dissection (200µm), C: Close-up of the region between Ci and C2 (100µm) D: close-up of C2 showing the difference between the AZ and MZ (50µm) E: MZ region showing compact cells with large nuclei and multiple nucleoli. (20µm). F: Close-up of AZ region with very little to no evidence of presence of cells. (20µm) UAG: Unpaired Exit duct, PAA: Paired Exit duct, C1: Constriction 1, C2: Constriction2, AZ: Anterior zone, MZ: Middle Zone, PZ: Posterior zone.

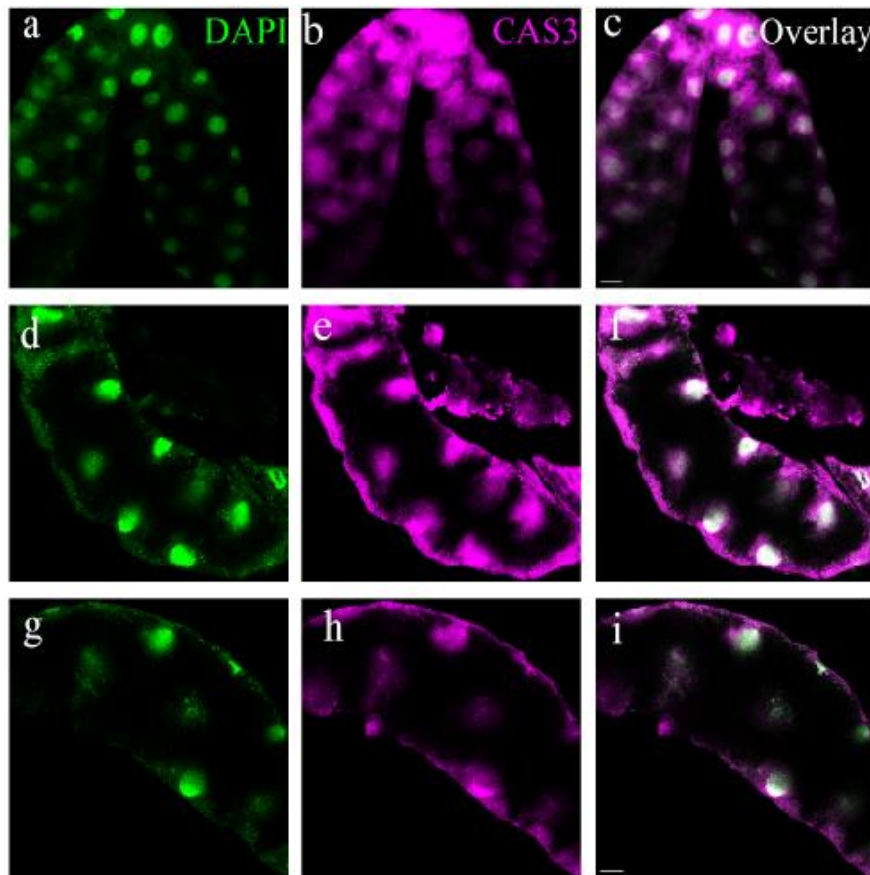


Figure 5. Caspase activity in larval whole mount salivary glands. A-C shows Day 8 glands (100 μ m), D-F shows day 9 salivary glands (50 μ m) and G-I shows day 10 salivary glands (50 μ m).

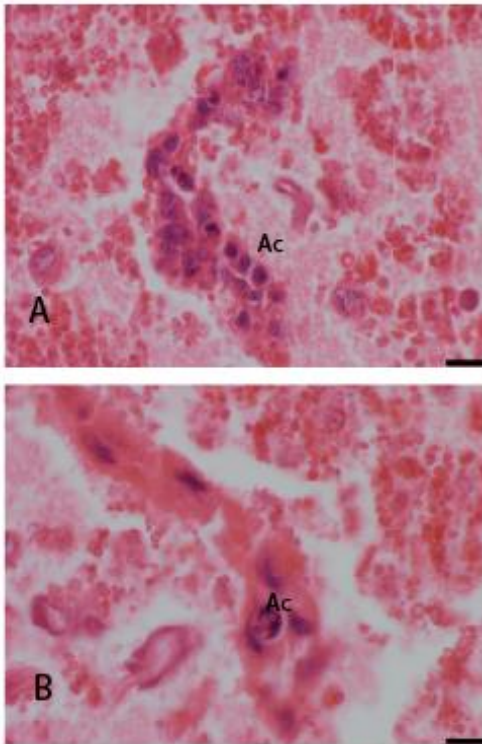


Figure 6. Apoptotic cells seen with hematoxylin and eosin stain in day 9 larval salivary gland. A: 50 μ m and B: 20 μ m. Ac: Apoptotic cells

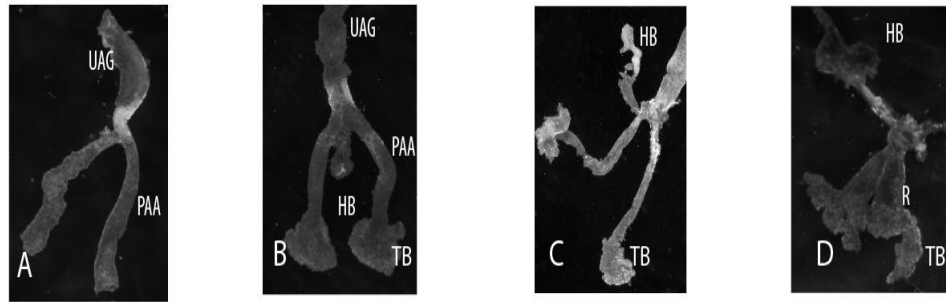


Figure 7. Development of the salivary glands during the pre-pupal stage. Evidence through Dissection. A: Day 12 (100µm), B: Day 12± 12 hours (100µm), C: Day 13± 12 hours (50µm), D: Day 13± 24 hours (50µm).

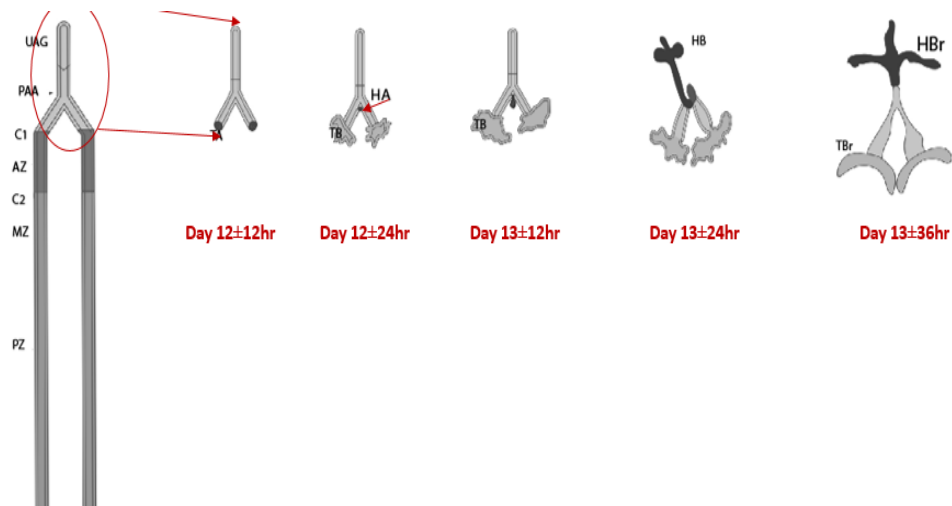


Figure 8: Plan of development of the pupal salivary from the remnants of the larval salivary. UAG: Unpaired Exit duct, PAA: Paired Exit Duct, AZ: Anterior Zone, MZ: Middle Zone, PZ: Posterior Zone, TA: Thoracic anlagen, TB: Thoracic Bud, TB_r: Thoracic branch, HA: Head anlagen, HB: Head Bud, HB_r: Head Branch

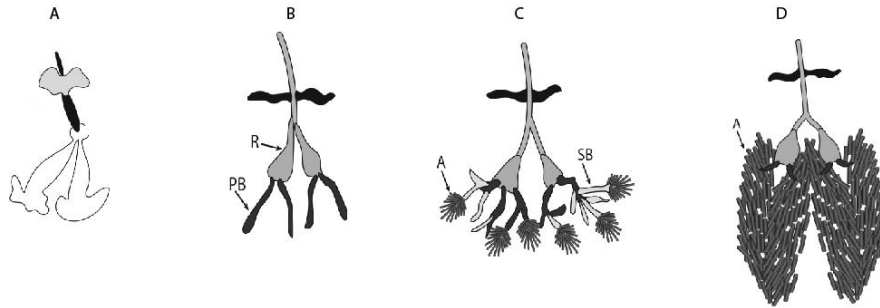


Figure 9. Illustration of development of the thoracic salivary during metamorphosis of honey bee. A: Day 12 \pm 12 hours, B: Day 14 \pm 24 hours, C: Day 15 \pm 24 hours, D: Day 16 \pm 12 hours. TB: Thoracic bud, R: Reservoir, PB: Primary branch, SB: Secondary branch, A: Acini.

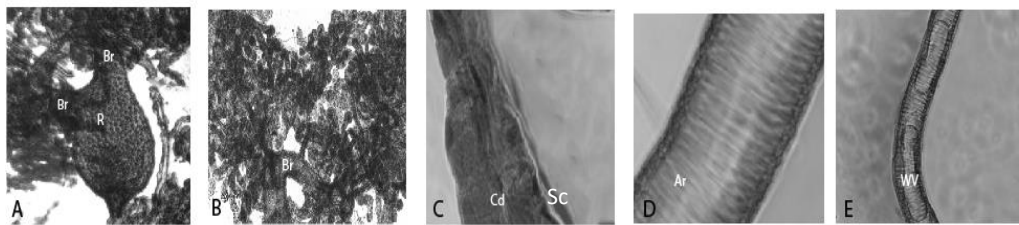


Figure 10. Thoracic Salivary. A: Reservoir of the thoracic gland showing the reservoir (R) and two main primary branches (Br), (100µm). B: Highly branched thoracic gland (100µm). C: Tubular acini's of the thoracic salivary showing the central duct (Cd) with the secretory cells (Sc) (20µm). D: Actin rings (Ar) in the primary branches (20µm). E: Water vacuoles (WV) inside the ducts. (50µm)

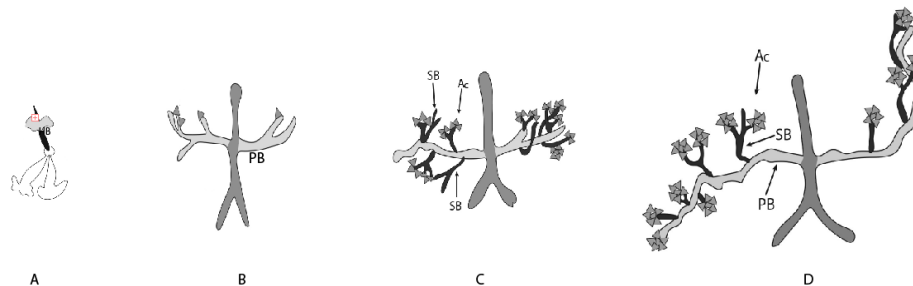


Figure 11: Illustration of the development of head salivary gland in honey bees. A: Day 12 \pm 12 hours salivary, B: Day 15 \pm 12 hours, C: Day 16 \pm 24 hours, D: Day 18 \pm 24 hours. HB: head bud, LB: Lateral Branches, PB: Primary branch, SB: Secondary Branch. Ac: Acini

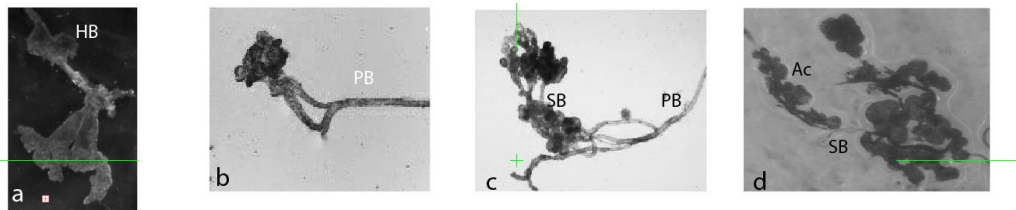


Figure 12. Development of the head salivary, evidence through dissection. A: Day 12 \pm 12 hours (50 μ m), B: Day 13 \pm 24 hours (100 μ m), C: Day 14 \pm 24 hours (100 μ m), D: Day 19 \pm 24 hours. (100 μ m). HB: Head bud, PB: Primary Branch, SB: Secondary Branch, Ac: Acini.

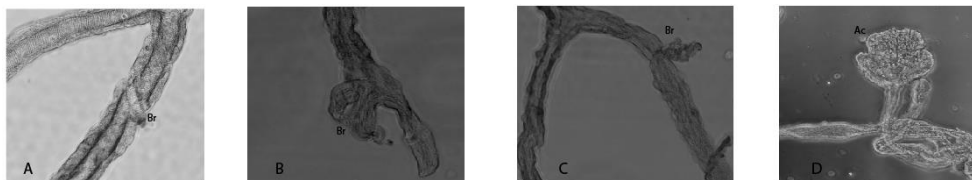


Figure 13: Branching events of the head salivary during Day 16 \pm 24 hours of metamorphosis. A, B and C show different branching events (20 μ m), D shows the

formation of the alveolar acini during Day 16±24 hours (20µm). Br: Branching, Ac: Acini.

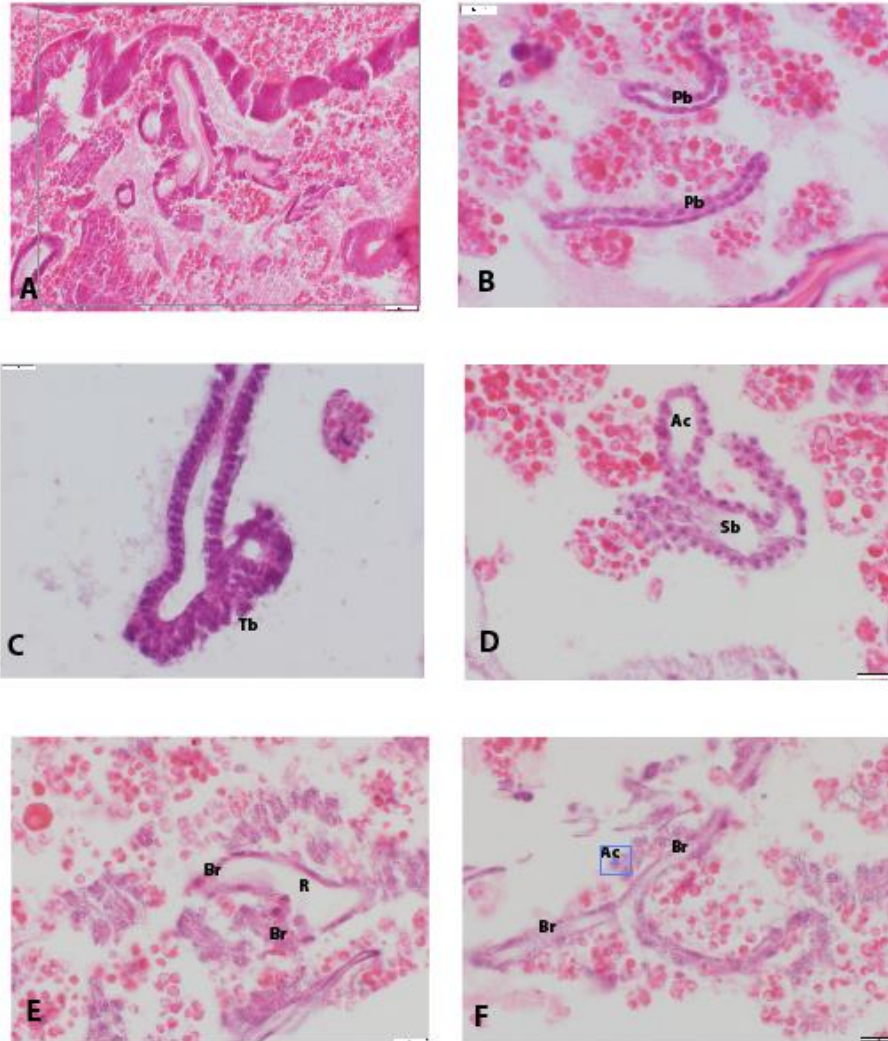


Figure 14: Histology of the head and thoracic salivary gland of pupal honey bee. A: Remnants of larval salivary, Day 12±24 hrs. (20µm). B: Primary head branches (Pb), Day 14±24 hrs. (50µm), C: Budding of the thoracic salivary (Tb) to initiate branching, Day 14±24 hrs. (20µm), D: Acini (Ac) formation and secondary branching (Sb) in the head salivary seen on Day 18±24 hrs. (50µm), E and F:

Reservoir(R), branches (Br) and acini (Ac) in thoracic salivary seen on day 16±24 hours (50µm).

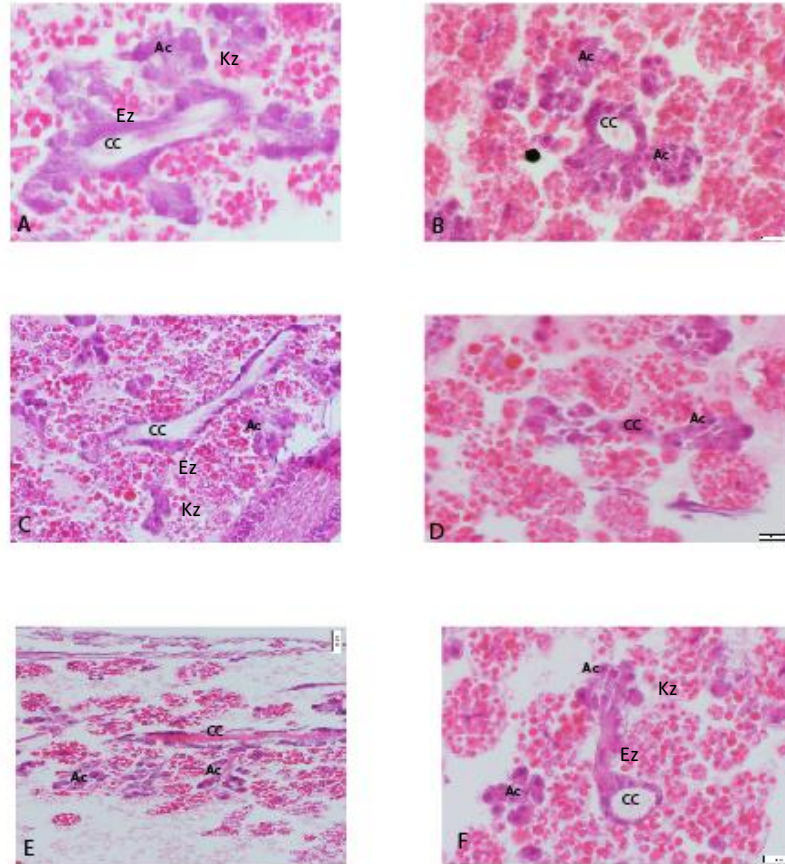


Figure 15. Histology of the hypopharyngeal gland development during pupation. Progression of extension of the canal cells are seen along with movement of the acini away from the central canal. A and B(20µm) are sections of the day 14 hypopharyngeal gland showing the acini (Ac)concentrated more around the central canal.(CC). C and D (50µm) showing thinning of the acini cells and also the increase in length in the duct cells of the gland in day 16 pupa. E and F (50µm) are

more like the adult glands wherein we see more thinning of the gland to give rise to a matured gland. On day 16 and 17, Cc: Central canal of the hypopharyngeal gland, Ac: Secretory acini, Kz, Secretory cells, Ez: Duct cell.

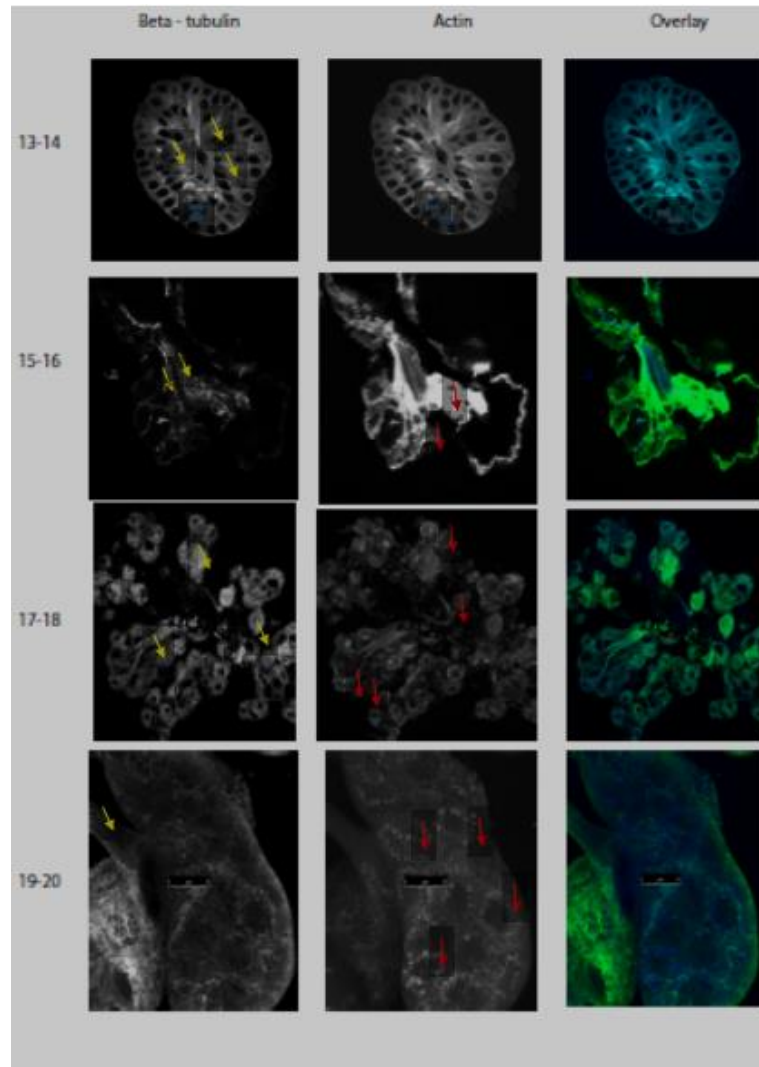


Figure 16: Agarose sections of the different stages of the hypopharyngeal glands during metamorphosis showing the microtubules and actin changes in the hypopharyngeal glands using tubulin and actin stains. ↘ = tubulin, ↓ = Actin, → = Proliferating cells.

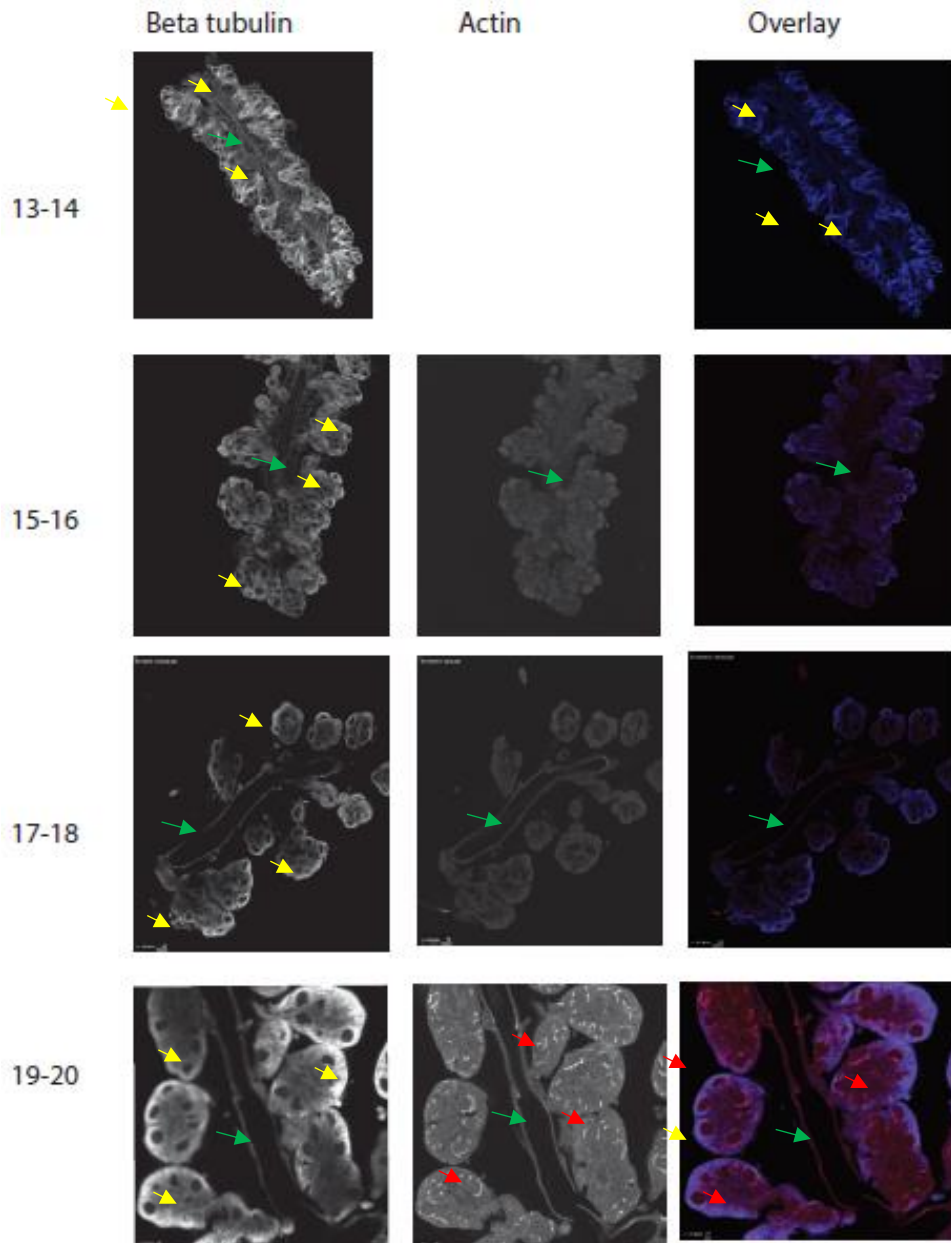





Figure 17: Whole mounts of the hypopharyngeal glands of different stages of metamorphosis showing portions of the complete gland and the changes in the gland morphology in relation to the migration of the acini's from the central duct.  = Central duct.  = Tubulin,  = actin.

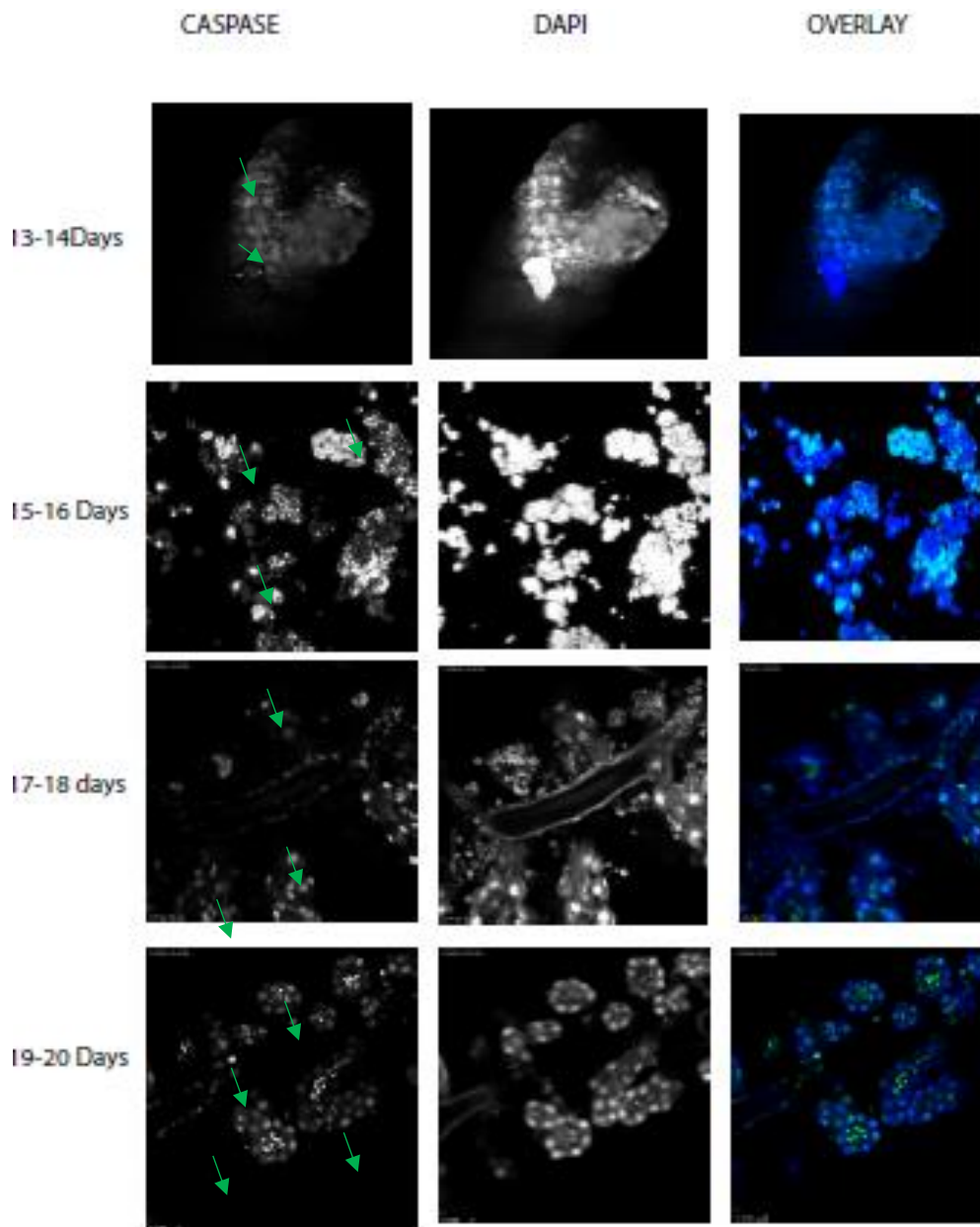


Fig 18: Different stages of the hypopharyngeal glands showing caspase activity, confirming the role of apoptosis throughout the maturation of hypopharyngeal glands from day 13 to day 20. 📌 = Apoptotic cells (Caspase 3)

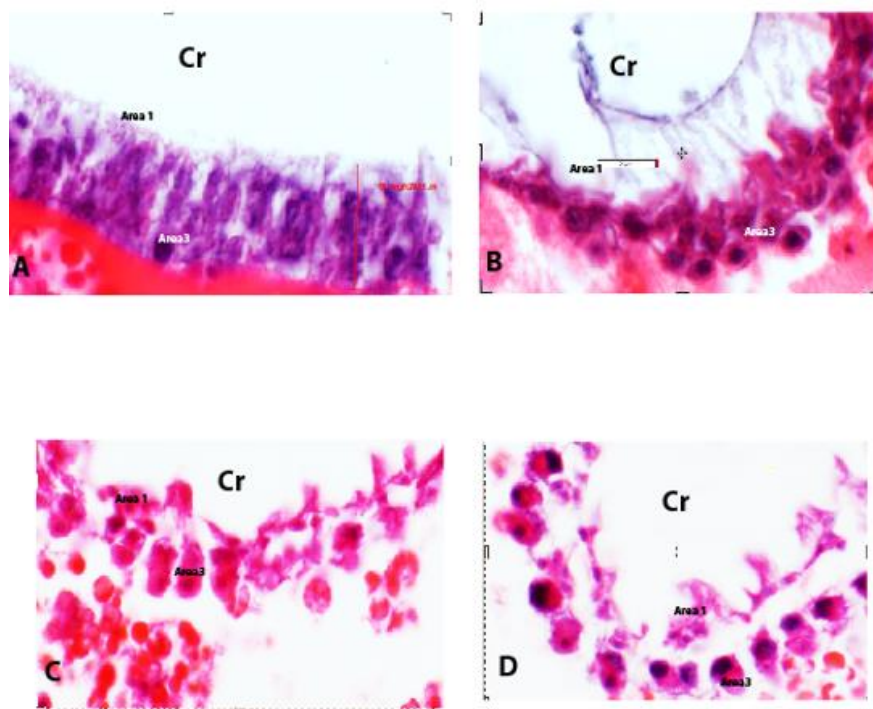


Figure 19: The morphological changes in the development of the mandibular gland during metamorphosis of honey bee larva. Cr: Central reservoir of mandibular gland, Area3: Secretory cells of the gland with spherical nucleus, Area 1: Stringy Cells that forms muscles.

Table 1: Mean \pm SD of the mandibular gland boundary diameter in different stages of the pupa

Pupa Age	Mandibular boundary diameter(μm)	No of samples(bees)
Day13-14	37.78 \pm 6.26	5
Day15-16	37.29 \pm 3.39	4
Day 17-18	35.68 \pm 0.355	5
Day 19-20	24.58 \pm 1.37	5

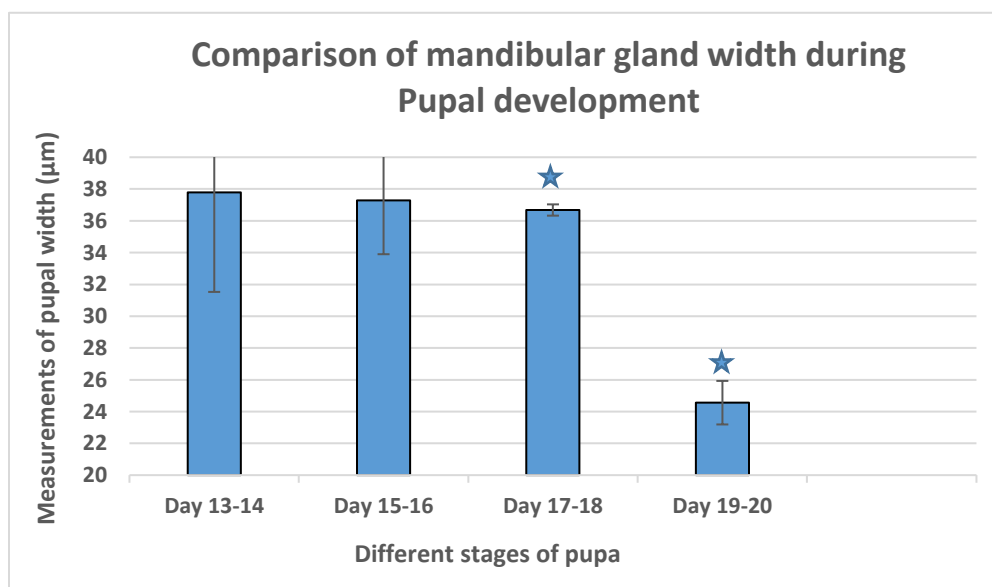


Fig 20: Diameter of the mandibular glands during pupal development shows continuous decrease in the width of the mandibular layer as it gets close to eclosion. Indicates a significant difference between stages ($P \leq 0.005$)

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