# Effect of cuticle surface and external structures of Lepidoptera larvae on entrapment of pesticide droplets

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# ABSTRACT

An insect's defence mechanisms vary during its different development stages. Certain stages are more susceptible to insecticides than others, with the cuticle that varies in its composition. Insects can also develop resistance to insecticides. The mechanisms of insect resistance include altered cuticles that reduce the penetration of insecticides, reduced sensitivity of the target site, and increased activity or level of detoxification of enzymes. Toxins are absorbed more slowly by insects that evolved penetration resistance compared to susceptible insects. This may be due to the outer cuticle that, for example, may be thicker in resistant insects, provide a barrier to insecticides. These barriers reduce the absorption of harmful chemicals into their bodies. A thickened cuticle layer could further delay penetration of insecticides. Wetting ability of the cuticle of insects is also important for effective control with spray applications of insecticides. Wettability is affected by the cuticle surface structure, as well as protuberances on the cuticle. The aim of this study was to examine the mesonotal cuticle thickness of different instar larvae of *Spodoptera frugiperda* (J.E Smith) (Lepidoptera: Noctuidae), as well as the cuticle surface and protuberances of *S. frugiperda, Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) larvae. The role of external cuticle structures in droplet entrapment was also observed. Third to sixth-instar larvae from the respective species were used in this study. Larvae were freeze-dried, after which the head and thorax were removed and prepared to capture micrographs of the pronotal surface appearances and to measure cuticle thickness using a scanning electron microscope (SEM). The cuticular surface topography differed between the species. Protuberances on the cuticle of *H. armigera* included large, closely spaced conical protuberances. The bumps present on the cuticular surface of *S. frugiperda* were absent on the cuticles of *H. armigera*, *C. partellus* and *B. fusca*. The cuticle of later instar *S. frugiperda* larvae (fifth- and sixth-instar) was significantly thicker than the cuticles of third- and fourth-instar larvae, which may contribute, together with other mechanisms, to older larvae being more tolerant to insecticides. The wax-coated spikes and/or bumps on the cuticle surface alter the hydrophobicity or hydrophilicity and allow non-waxy structures to efficiently capture water droplets. Third and sixth-instar larvae from the respective species were sprayed with water, as well as water containing a non-ionic wetting agent. Stereomicroscope images showed that droplets were entrapped by the roughness of the cuticle, as well as by setae on the cuticle surface. Surface hydrophobicity was reduced when a surfactant was added to the spray mixture. It is therefore important to adhere to label recommendations of insecticides and use the correct nozzle to obtain droplets of the recommended size for effective covering of lepidopteran pest larvae. The addition of a wetting agent was also observed to increase wettability and its addition to spray mixtures may improve effective covering of the cuticle of the target insect.

**Key words:** acanthae, conical-shaped, droplet size, non-ionic wetting agent, nozzle, protuberances, superhydrophobicity.

## GLOSSARY

Absorption: Intussusception of fluid by living cells or tissues; passage of nutritive material through living cells.

Acantha: prickle or spinous process.

Adhesion: condition of touching without growing together of parts normally separated, as between members of different series of floral leaves.

Arthropoda: a phylum of metamerically segmented animals with jointed legs and a thickened chitinous cuticle forming an exoskeleton, and having haemocoel, head, and sometimes a telson.

**Biofilm: a thin but robust layer of mucilage adhering to a solid surface and containing a community of bacteria and other microorganisms.

Chaetae: chitinous bristle, as of certain annelids, embedded in the body wall; seta

Chitin: a linear array of β-linked N-acetyl-glucosamine units, a mucopoly-saccharide, found in annelid cuticle and arthropod exoskeleton and in some plants.

Conical: having the shape of a cone.

Cuticle: an outer skin or pellicle; a layer of material laid down over the epidermis in animals.

Dorsal: back, or laying near back.

Endocuticle: the elastic inner layer of insect cuticle.

**Entrapment: the state of being caught in or as in a trap.

Epicuticle: lamella or membrane external to exocuticle.

Epidermis: single layer of ectoderm in invertebrates.

Exocuticle: middle layer of insect cuticle, between endocuticle and epicuticle.

Exoskeleton: a hard supporting structure secreted by and external to ectoderm or skin.

Hair: any epidermal filamentous outgrowth consisting of one or more cells, varied in shape.

Hydrophile: adsorbing water.

**Hydrophobe: tending to repel or fail to mix with water.

*Integrated pest management (IPM): a broad-based approach that integrates practices for economic control of pests. IPM aims to suppress pest populations below the economic injury level (EIL).

Integument: a covering structure or layer.

Larva: an embryo which becomes self-sustaining and independent before it has assumed the characteristic features of its parents.

Lepidoptera: an order of insects including butterflies and moths, having complete metamorphosis, two pairs of membranous wings covered with scales, a sucking proboscis, and caterpillars as larvae.

Microfibrils: small fibrils composed of chains of cellulose molecules, visible only with the electron microscope, and aggregated to form macrofibrils.

Microtrichium: one of the small hairs without basal articulations in insect wings.

Polyphagous: insects using many different food plants.

Procuticle: the colourless cuticle of insects, composed of protein and chitin, before differentiation into endocuticle and exocuticle.

Prothorax: the anterior segment of the thorax of an insect.

**Protuberances: a structure that protrudes from something else.

*Resistance: the act or power of resisting, opposing, or withstanding

Scale: a flat, small, plate-like external structure, dermal or epidermal; a chitinous outgrowth.

Sclerotization: the process of hardening and darkening the exoskeleton which occurs in insects after ecdysis.

*Sensory cell: a nerve cell that conducts impulses from a sense organ to the central nervous system.

Seta: a bristle-like structure; extension of exocuticle, produced by trichogen: a hair, bristle, or scale of insects.

Spine: a sharp-pointed process.

**Superhydrophobic: tending not to dissolve in, mix with, or be wetted by water

*Surfactants: a substance which tends to reduce the surface tension of a liquid in which it is dissolved.

Trichogen cell: a seta-producing cell in some arthropods.

Unicellular: consisting of one cell.

*Volume median diameter (VDM): refers to the midpoint droplet size (median), where half of the volume of spray is in droplets smaller and half of the volume is in droplets larger than the median.

***The glossary was compiled from:***

Holmes, S. 1986. Henderson's Dictionary of Biological Terms, Ninth Edition.

*O'Neill, M., Summers, E., and Collins, W.1979. Collins English Dictionary, Thirteen Edition.

**Oxford languages. 2010. Oxford dictionary of English, Third Edition, Oxford University Press.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 Maize production

Maize is an important staple food crop in African countries. It is the most important cereal crop in terms of food security, and used by 50% of the more than 300 million people in sub-Saharan Africa (Badu-Apraku and Fakorede, 2017). Maize is also the most informally traded staple product in southern Africa (Mango *et al*., 2018), and a major food and feed crop in South Africa (Greyling and Pardey, 2019). South-Africa is one of the top maize producers, ranked 9th in the world (Badu-Apraku and Fakorede, 2017). A crop of 16,211 million tons was produced on 2 610 800 ha in the 2021 production season (Crop estimates committee, 27 October 2021).

Current crop losses inflicted by insect pests in Africa are estimated at 49% of the total crop yield each year. Maize hosts various lepidopteran pests. The economically most important species in sub-Saharan Africa are the stem borers, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae), *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Kfir *et al*., 2002; Oben *et al*., 2015; Sokame *et al.*, 2020). Two other important lepidopteran pests of maize are the African bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), attacking both the leaves and ears of maize plants (Van den Berg *et al*., 2015), and the Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Goergen *et al*., 2016). Larvae cause damage by feeding on all above-ground plant parts and during all growth stages of maize plants (Al-Sarar *et al.*, 2006).

## 1.2 Insect pests

*Chilo partellus* (Fig. 1.1a) and *B. fusca* (Fig. 1.1b) are destructive lepidopteran pests of maize in Africa (Calatayud *et al*., 2014). Yield of maize crops is negatively affected when stem borers continuously infest the crop throughout the growing season (Kfir *et al*., 2002; Oben *et al*., 2015; Sokame *et al.*, 2020). Plant damage resulting in 10-100% yield loss by these stem borers has been reported by Kfir *et al.* (2002). Lepidopteran stemborer species may occur as single species or as a community of mixed species (Van den Berg *et al*., 1991; Tefera, 2004). The crop can be affected by these pests from the seedling stage to harvesting (Peddakasim *et al*., 2018). They can be detected through characteristic feeding holes in the leaves and the presence of larval entry holes in the stems (Peddakasim *et al*., 2018). Young *C. partellus* and *B. fusca* larvae feed inside the whorls of plants, and older larvae tunnel into stems, causing destruction of stem tissue (Nabeel *et al.*, 2018). Once stemborer larvae enter into stems, they cannot be affected by insecticide applications, which impedes chemical control strategies (Kfir *et al.*, 2002; Oben *et al.* 2015; Nabeel *et al.*, 2018; Rodriguez *et al*., 2018; Hardwick *et al.*, 2019).

*Chilo partellus* moths lay yellowish, disc-shaped eggs in batches of approximately 30 eggs. Eggs hatch in 5-7 days, while the larval stage lasts 3-4 weeks, during which time it develops through six instars. The larvae are creamy-white to yellowish-brown in colour, with several conspicuous dark brown spots on each segment. Two to three setae are found in each spot and are more prominent in older larvae (Kalaisekar *et al.*, 2016). Larvae grow to about 30 mm in length (Van den Berg and Van Rooyen, 2014).

*Busseola fusca* moths lay approximately 200-300 eggs under field conditions. The eggs are spherical, and the chorion has radial ridges, initially creamy-white in colour and darkening with age. Neonate larvae are dark brown, becoming paler as they mature. Fully grown larvae are 35-40 mm long. *Busseola fusca* larvae feed inside maize whorls for 10-20 days before they leave and enter the stems, where they feed until fully grown. The larvae moult 5-6 times and the pupal stage lasts 2-3 weeks, with the overall duration of the life cycle being approximately nine weeks (Van den Berg *et al*., 2015).

The bollworm, *H. armigera* (Fig. 1.1c), a polyphagous pest of crops globally, is known to adapt easily to different environments (Yucel and Genc, 2018). A variety of plant families, including many crops of economic importance, such as tomato, maize, cotton, sorghum and soybean among others, are damaged by *H. armigera* larvae (Yucel and Genc, 2018). *Helicoverpa armigera* feeds on various parts of the plant, but prefers to eat the reproductive organs (Fitt, 1989). Damage to the silk of young maize ears is common. When silks are severely damaged, pollination cannot occur, causing direct yield loss (Van den Berg and Van Rooyen, 2014). Tassel and ear damage by *H. armigera* larvae, is also typical for this species (Keszthelyi *et al.*, 2011). Larvae of this pest can be found in high numbers if not controlled effectively (Wondafrash *et al.*, 2012).

*Helicoverpa armigera* moths lay on average between 730 and 1600 eggs during a lifespan of 2-3 weeks. The eggs are shiny and yellowish-white after oviposition, but turn dark brown before they hatch. Neonate larvae are 1.5 mm in length, while the length of fully-grown larvae ranges between 28-40 mm. There are five to six larval instars and the entire larval stage lasts for 2-3 weeks, depending on temperature (Bennet, 2019).

*Spodoptera frugiperda* (Fig. 1.1d) is native to the Americas, but the first outbreaks of this pest on the African continent were reported in West and Central African countries during 2016 (Goergen *et al*., 2016). The estimated maize yield losses caused by this pest in 12 of Africa's maize-producing countries, in the absence of any control methods, range from 8.3 to 20.6 m tonnes per annum. The value of these potential losses is estimated at between $2,481 m and $6,187 m (Day *et al*., 2017; Nagoshi *et al.,* 2018; Shylesha *et al.,* 2018).

*Spodoptera frugiperda* is highly polyphagous, with 353 larval host plants belonging to 76 plant families (Montezano *et al*., 2018). The most severe damage is, however, caused to grasses such as maize and sorghum, and also to cotton and soybean (Pitre and Hogg, 1983; Hardke *et al*., 2015). Young leaves, leaf whorls, tassels and the ear of maize plants are damaged by *S. frugiperda* larvae (Al-Sarar *et al.*, 2006). Early in 2017, damage to maize crops was reported in the Limpopo and North West provinces in South Africa (Jacobs *et al*., 2018).

The larval stage of *S. frugiperda* consists of six instars. Depending on temperature and other environmental conditions, the duration of the larval stage can range between 12 and 20 days. Different instar larvae vary in length from 1.7-34.2 mm (Capinera, 2000). Larvae can be identified by four characteristic dark dots, arranged in a square, on the second last segment of the larvae. The head, dark in colour, displays an inverted Y-marking on the head capsule (Fig. 1.1d) (Hardke *et al*., 2015).

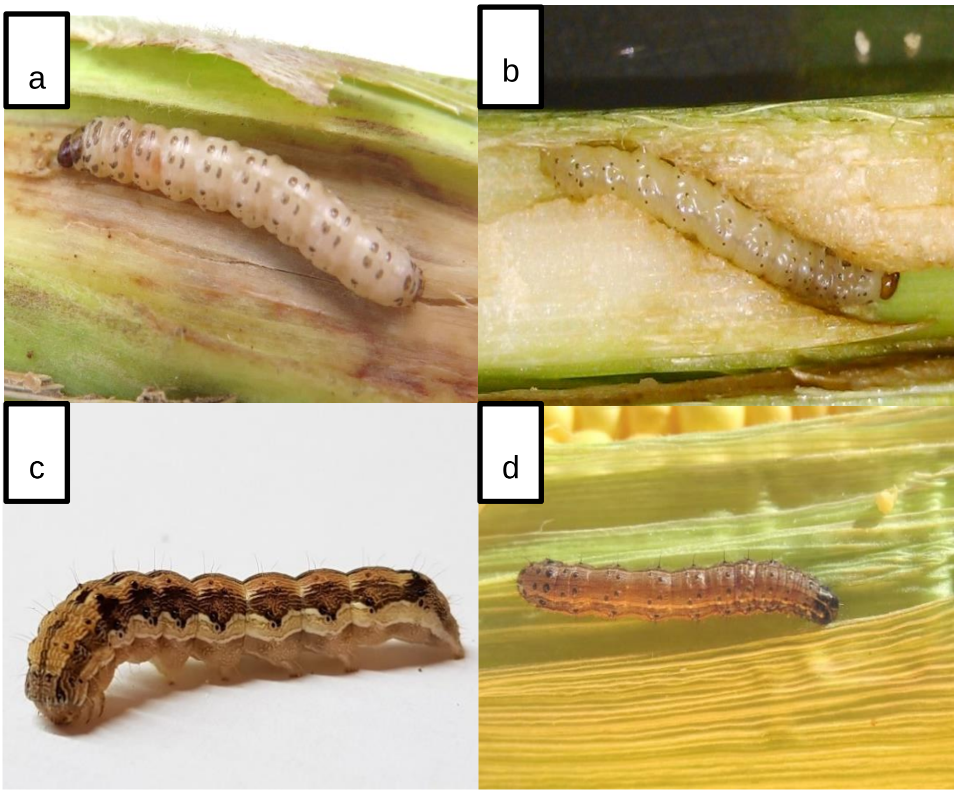

Figure 1.1 Lepidopteran pests of maize in South Africa. Final instar larvae of: (a) *Chilo partellus,* (b) *Busseola fusca,* (c) *Helicoverpa armigera*, (d) *Spodoptera frugiperda*.

### 1.3 Control of lepidopteran pests

Pesticides play a fundamental role in controlling pests (Pimentel and Levitan, 1986) and contribute to the increase in agricultural production of crops (Schwinn, 1988). Inorganic pesticides were introduced in the early 20th century (Felsot and Rack, 2007). The synthetic organic insecticides (cyclodienes and organophosphorus insecticides) were introduced in the 1940s and improved the insecticidal efficacy and spectrum (Sparks and Nauen, 2015). A concurrent increase in the use of these insecticides was also recorded (Sparks and Nauen, 2015). Since the introduction of synthetic organic insecticides, control of insects was based on their use (Luck *et al.*, 1977) and they have also become the primary pillar in crop management (Schwinn, 1988). From the total amount of insecticides applied, 80% are applied onto crops such as maize, sorghum, rice, cotton, vegetables and fruit (Schwinn, 1988). The primary method for control of maize pests is by means of insecticides (All *et al*., 1989) and, according to Meissle *et al*. (2010), pesticides are used increasingly to control maize pests. Justification for the use of insecticides in agriculture, is the convenience of use, quick results (since they are fast acting), and insecticides are relatively inexpensive (Zhu *et al.*, 2016). Control measures that are fully dependent on insecticides often result in pests developing resistance to major classes of these insecticides (Yu, 1992). Insecticide resistance has therefore been a major factor influencing insect control and pest management for more than half a century (Sparks and Nauen, 2015).

The pest complex on each crop requires an unique mixture of pesticides (Quinn *et al.*, 2011). Chemical control of stem borer species was intensively studied in South Africa in the 1990's (Van Rensburg, 1990; Van Rensburg and Van den Berg, 1992; Van den Berg and Van der Westhuizen, 1995). This was before the introduction of Bt maize for control of these stem borers, in the 1998/99 season (Gouse *et al.,* 2005). Although insecticide applications for the control of stem borers on maize were then reduced (Gouse *et al*., 2005), applications are still being done on the non-Bt maize refuges planted next to Bt maize. Kruger *et al*. (2011) and Van den Berg *et al*. (2013) reported that farmers on the eastern Highveld region of South Africa apply insecticides preventatively on both Bt maize and the non-Bt maize refugia.

Bollworm outbreaks on maize are sporadic and only occur in isolated areas (Van den Berg *et al*., 2015). Long term application of insecticides for control of *H. armigera* on maize is therefore not advised, but should be planned properly (Van den Berg *et al*., 2015). *Spodoptera frugiperda* is, however, mainly controlled with insecticides on maize (Yu, 1983; Carvalho *et al*., 2018; Sisay *et al.*, 2019).

Larvae of different lepidopteran maize pests are often controlled with the same active ingredient or insecticide group (Table 1.1) (Agri-Intel, 2020). Larvae from different lepidopteran species may also occur simultaneously on a single maize plant. FAW co-exists with other stem borer species on maize plants in Africa (Sokame *et al*., 2020). It has implications in terms of control, also. For example, if an insecticide is applied against *C. partellus*, while it is still in the whorl of a maize plant, larvae from the other pest(s) will also be controlled. Some of the insecticides registered for stem borer control in South Africa, are also registered against *H. armigera* and *S. frugiperda*, for example, methomyl (Table 1.1).

Table 1.1 Chemical group and active ingredient of insecticides registered for control of *Chilo partellus*, *Busseola fusca*, *Helicoverpa armigera* and *Spodoptera frugiperda* in South Africa (Summarized from Agri-Intel, 2020).

| **Chemical group** | **Active ingredient** | **Lepidopteran species** | | | |
|---|---|---|---|---|---|
| | | ***Chilo partellus*** | ***Busseola fusca*** | ***Helicoverpa armigera*** | ***Spodoptera frugiperda*** |
| **Pyrethroids** | *alpha*-Cypermethrin | | X | X | |
| | Fenvalerate | | X | | X |
| | *beta*-Cyfluthrin | X | | X | |
| | Bifenthrin | | X | X | |
| | *lambda*-Cyhalothrin | X | X | X | X |
| | Cypermethrin | | X | X | |
| | *beta*-Cypermethrin | | | | X |
| | Deltamethrin | X | X | X | |
| | Esfenvalerate | | X | X | |
| | *gamma*-Cyhalothrin | X | X | X | |
| | *zeta*-Cypermethrin | | | X | |
| **Carbamates** | Benfuracarb | | X | | X |
| | Carbosulfan | X | X | | X |
| | Thiodicarb | | X | | |
| | Methomyl | | | X | X |
| **Diamides** | Chlorantraniliprole | X | X | | X |
| | Flubendiamide | | | | X |
| **Organophosphates** | Chlorpyrifos | | X | | X |
| | Malathion | | | | X |
| **Oxadiazines** | Indoxacarb | | X | X | X |
| **Benzoylureas** | Lufenuron | | X | | X |
| | Novaluron | | | X | X |
| **Diacylhydrazines** | Methoxyfenozide | | X | | |
| **Spinosyns** | Spinetoram | | X | | X |
| **Avermectin** | Emamectin benzoate | | | X | X |
| **Compounds of unknown or uncertain MoA** | Pyridalyl | | | | X |
| **Baculoviruses** | Nucleopolyhedrovirus (*Helicoverpa armigera*) | | | X | |
| **Microbial disruptors of insect midgut membranes** | *Bacillus thuringiensis* | | | | X |
| **Fungal pathogen** | *Beauveria bassiana* | | | | X |
| **Combinations** | Chlorantraniliprole + lambda-cyhalothrin | X | X | X | X |
| | Bifenthrin + novaluron | | | X | |
| | Benfuracarb + fenvalerate | | X | | X |
| | Clorpyrifos + lambda-cyhalothrin | | X | | X |
| | Indoxacarb + novaluron | | | | X |
| | Methoxyfenozide + spinetoram | | X | | X |
| | Indoxacarb + lambda-cyhalothrin | | X | | |

The Insecticide Resistance Action Committee (IRAC) classified insecticides into more than 25 Modes of Action (MoA's) groups in 2015. However, 85% of the value of these MoA groups was contributed by insecticides with a MoA that acts on the insect nerve and muscle systems (Fig. 1.2) (Sparks and Nauen, 2015).

Insecticides altering growth and development accounted for 9% of the total insecticide sales, while those disrupting energy production (respiration targets) accounted for only 4% (Fig. 1.2) (Sparks and Nauen, 2015). Nerve and muscle targeting insecticides are generally fast acting (IRAC, 2020). The two growth regulators currently registered against lepidopteran pests in South Africa, *viz*. lufenuron and novaluron, are both benzoylureas (Table 1.1). Benzoylureas act primarily at sites of chitin synthesis in epidermal cells (Cohen, 1987; Mondal and Parween, 2000). Larvae of *S. frugiperda* and *H. armigera* are known to be affected by benzoylureas when ingested or it also acts as a contact insecticide (Ishaaya, 2011). Early corrective treatment with diamides, targeting the ryanodine receptors and calcium channels in insect muscles, is recommended for *B. fusca* and other stem borer larvae on maize crops (Rani *et al.,* 2018).

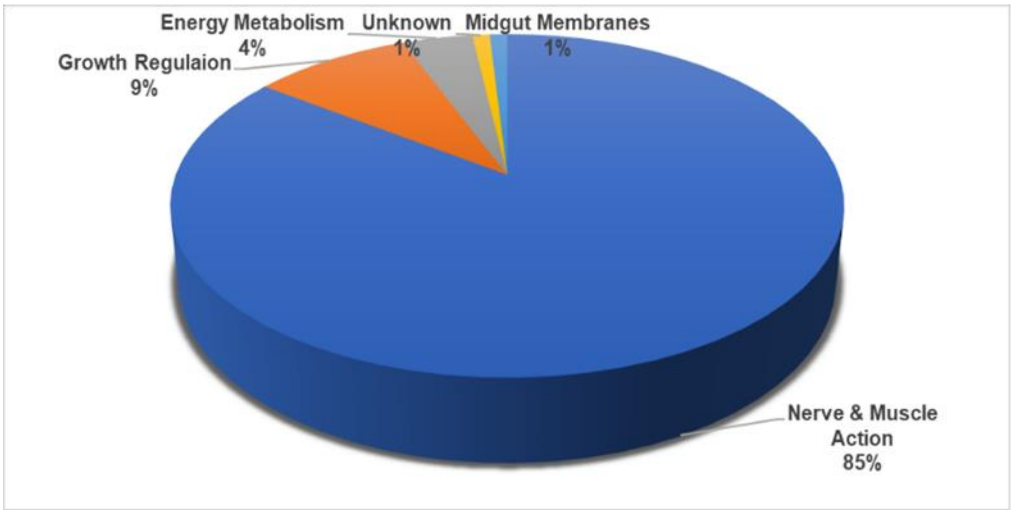

Figure 1.2 Distribution of total insecticide sales (percent of total value) according to broad mode of action. Total value - $17 016 million; excludes fumigants. Based on 2013 End-user sales data from Agranova (From Sparks and Nauen, 2015).

### 1.4 Evolution of resistance to insecticides

Insecticide activity is the result of a series of complex actions and counteractions between a toxicant and the tissue of an insect (Sun *et al.*, 1969). Resistance can be described as a change in the sensitivity of a pest population because of repeated failure of an insecticide that previously achieved expected levels of control when it was used (Zhu *et al*., 2016). Continued evolution of resistance by insects to insecticides hampers effective management of insect pests (Al-Sarar *et al*., 2006). Resistance evolves when insecticides are applied too often over consecutive seasons and/or in improper quantities (Al-Sarar *et al*., 2006). Globally, *H. armigera* has been controlled with insecticides from various groups for many years, resulting in a history of insecticide resistance that goes back several decades (Torres-Vila *et al*., 2002). *Spodoptera frugiperda* resistance to insecticides from various groups has also been reported (Nascimento *et al.,* 2016; Okuma *et al.,* 2018; Gutiérrez-Moreno *et al.*, 2019). After the introduction of synthetic insecticides in the late 1940's, the number of cases of insecticide resistance, and the number of species and compounds involved has continually increased (Fig. 1.3).

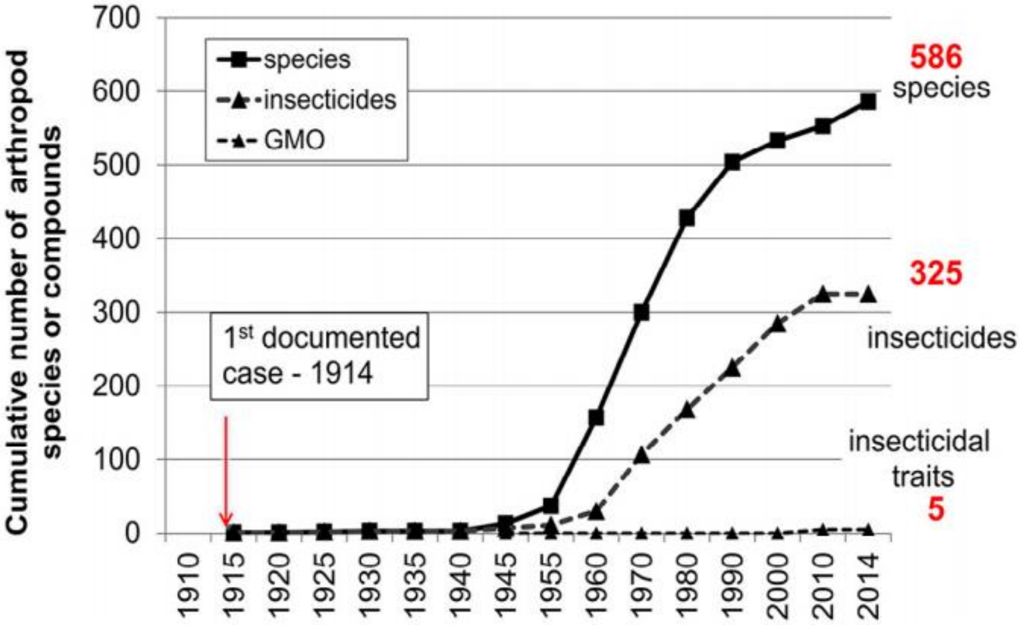

Figure 1.3 Cumulative increase in the number of species resistant to one or more insecticides, number of insecticides for which one or more species has shown resistance, and number of GMO traits for which resistance has been reported (From Sparks and Nauen, 2015).

There are three major insecticide resistance mechanisms in insects, namely reduced sensitivity of the target site, reduced penetration of the insecticide and increased activity or level of detoxification enzyme(s) (Kasai *et al.,* 2014). Modifications of the insecticide target protein, as well as metabolic resistance, are the best studied resistance mechanisms (Balabanidou *et al.*, 2018). The target site of insects that evolved target site resistance, is genetically modified so that the interaction and binding of an insecticide at this site, are prevented (Bass *et al*., 2011). Metabolic resistance involves the over-expression or higher catalytic activity of detoxifying enzymes responsible for inactivation of the insecticide by metabolism or sequestration (Balabanidou *et al.*, 2018). Resistant insects will therefore detoxify (metabolize) the toxin faster or will more quickly get rid of toxic molecules in their bodies, compared to susceptible insects (Panini *et al*., 2016).

The mechanism for penetration resistance is less studied but occurs when toxins are absorbed slower by resistant compared to susceptible insects. The outer cuticle of resistant insects develops barriers that reduce the absorption of the harmful chemicals into their bodies (Karaagac, 2012). Modification of the composition of the cuticle or increased thickness, mainly through enhanced deposition of structural components, such as epicuticular lipids and/or structural proteins, are responsible for the reduced penetration of insecticides into the body (Fang *et al*., 2015; Balabanidou *et al*., 2016). A thickened cuticle layer in resistant strains of insects could therefore delay penetration of insecticides (Tak and Isman, 2015). A slower rate of penetration allows detoxification enzymes more time to act, thereby multiplying their effect (Balabanidou *et al*., 2016). An example of penetration resistance was provided by Balabanidou *et al.* (2018), who reported on the penetration of deltamethrin into larvae of a susceptible *H. armigera* strain. Penetration of the produce occurred within an hour, while absorption of the insecticide by a resistant strain, took 6 hours. The respective mechanisms of resistance are illustrated in Figure 1.4, comparing a susceptible and resistant insect, respectively.

Insects, are small in size, resulting in high ratios of surface area to volume, and short pathways to the nervous system (Lewis, 1980). Insects are therefore vulnerable to contact insecticides that enter through the integument (Lewis, 1980). The first stage of the action of an applied insecticide, is the accumulation of the toxicant by the insect (Lewis, 1980). This process is determined by the behaviour of the insect, but also by the physical properties of the insecticide, in particular, the particle or droplet size (Lewis, 1980). The second stage is penetration of the insecticide through the integument of the insect.

Factors affecting penetration through an insect cuticle, include surface migration, diffusion across the epicuticular wax layers and the procuticle matrix, present below the lipid epicuticle (Lewis, 1980).

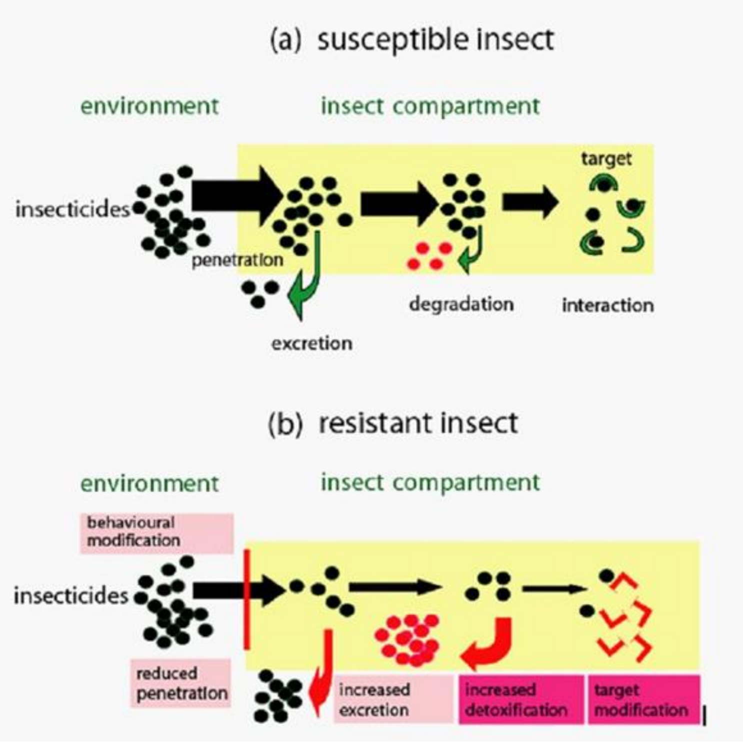

Figure 1.4. Mechanisms of resistance of an insect susceptible to insecticides compared to a resistant insect. More than one type of resistance mechanism may be present in insecticide resistant insects (From Corbel and N'Guessan, 2013).

## 1.5 The insect cuticle

The outer layer of an insect, referred to as the integument, consists of the epidermis and the extracellular cuticle (Chapman, 1998). The cuticle of insects is a very versatile structure (Fabritius and Moussian, 2017). The success of insects as terrestrial animals can be ascribed to the unique characteristics and protection provided by their cuticles (Chapman, 1998). It not only represents the interface of insects with their environment, but also covers the whole outside of the body and is diverse in its permeability and mechanical properties (Evans, 1984). It shapes and supports the insect body, provides a means of locomotion, serves as a temporary food storage, and it is also a major barrier to parasitism, disease and water loss (Vincent and Wegst, 2004). The cuticle may be transparent, or non-transparent, and rigid or elastic (Evans, 1984).

Formation of the cuticle is the result of proteins, lipids, carbohydrates and water that are excreted by a single layer of epidermal cells, the epidermal sheet (Evans, 1984). The cuticle is usually unmodified, and secretions reach the external surface of the cuticle by means of pore canals and epicuticular filaments (Chapman, 1998). It covers the entire surface of the insect and extends into the tracheal system, fore- and hind-gut, as well as parts of the genital system (Vincent and Wegst, 2004).

Insect cuticles are commonly divided into several layers, with from the outside, cement and wax (Vincent and Wegst, 2004), followed by the three main layers, *viz*. the epi-, exo- and endocuticle (Vincent and Wegst, 2004, Mitov *et al*., 2018). The epicuticle is rich in lipids and proteins but contains no chitin and forms the thin outer region (Chapman, 1998; Evans, 1984). It consists of a very thin, tough, insoluble, non-elastic, approximately 15 nm thick outer epicuticle, followed by the inner epicuticle, which is between 0.5 and 2.0 µm thick (Chapman, 1998; Evans, 1984). The epicuticle can be smooth or sculptured and extends into surface structures such as fine protuberances (Resh and Cardé, 2009). Cuticle surfaces that are sculptured into fine parallel grooves can act as diffraction gratings (Resh and Cardé, 2009).

Underneath the epicuticle, and forming the bulk of the cuticle, is the procuticle, which consists of the exo- and endocuticle (Gillott, 2005). Several very fine pore canals transverse through the endo- and exocuticle and branch into numerous finer wax canals within the epicuticle. This system is responsible for the transportation of lipids (waxes) from the epidermis to the epicuticular surface (Gullan and Cranston, 2010). Insects that lack a cement layer, which covers the wax layer and protects it from abrasion (Holmes, 1986), produce a wax layer that consists of a complex mixture of lipids, which forms the outermost layer of the epicuticle (Mitov *et al*., 2018).

The wax layer of the epicuticle (Fig. 1.5b) determines many of the surface properties and it is also the barrier between insect tissues and the outside world (Chapman, 1998; Evans, 1984). This layer prevents water loss and desiccation, and contributes to the superhydrophobic and self-cleaning properties of the cuticle (Locke, 1961).

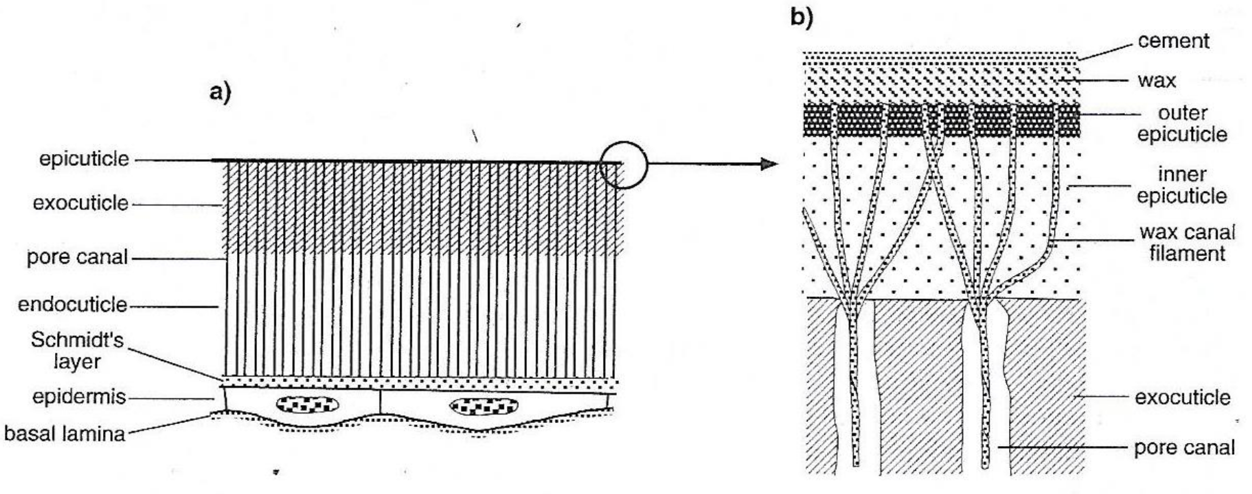

Figure 1.5 Basic structure of the insect integument: a) section through a mature integument, and b) section through the epicuticle at greater magnification (From Chapman, 1998).

The characteristic component of the exo- and endocuticle is chitin, a polysaccharide largely consisting of N-acetylglucosamine residues and some glucosamine molecules (Chapman, 1998). It also contains carbohydrates and proteins (Chapman, 1998; Evans, 1984). After secretion of the procuticle during moulting, the outer region darkens and becomes hard and rigid (tanned or sclerotized) due to the deposition of sclerotin to form the exocuticle, whereas the undifferentiated region is lighter in colour and softer, which is referred to as the endocuticle (Fig. 1.5a) (Wigglesworth, 1948; Hackman, 1953; Chapman, 1998; Evans, 1984; Beutel *et al.*, 2014). A hardened but not fully darkened layer, the mesocuticle, may form in the middle of the two regions (Chapman, 1998).

Chitin is a colourless, insoluble polysaccharide, which forms a long chain with the sugar residues all oriented in the same direction (Chapman, 1998). Chitin chains are held together by hydrogen bonds and form microfibrils that lie parallel in the cuticle, forming an embedded protein matrix (Evans, 1984). The microfibrils in the exocuticle rotate anticlockwise, performing a helicoidal arrangement to produce a series of thin lamellae called the lamellate cuticle (Chapman, 1998). Growth layers such as those produced in the cuticle during daytime are uniformly orientated, while those laid down at night have microfibrils arranged in a helicoidal pattern (Chapman, 1998).

There are a hundred or more different proteins present inside the insect body and their distribution depends on their location. For example, some proteins are associated with membranous regions of the cuticle, while others characterize the hard cuticle (Chapman, 1998). The different physical properties of different parts of the cuticle are therefore the outcome of the different proteins they contain (Chapman, 1998). Proteins produce a matrix of mechanical properties, also including interaction with and stabilizing the chitin (Vincent and Wegst, 2004). The forming of a rigid matrix by the cross-links between protein molecules, is a process called tanning or sclerotization, which is associated with the hardening of the cuticle (Chapman, 1998).

The structures or outgrowths that occur on cuticles are called protuberances and can be classified into four fundamental types: (1) multicellular, cells similar in appearance to those of the general epidermis cells, (2) multicellular, with specifically differentiated cells such as a seta, (3) unicellular, and (4) subcellular, or more than one projection per cell (Richards and Richards, 1979; Gullan and Cranston, 2010). The exoskeleton is mostly covered with these protuberances, of which the architecture ranges from relatively flat scales to long hairs and brush-like structures with complex geometry (Vincent and Wegst, 2004). The cuticle surface may, however, also have a smooth, embossed or engraved appearance (Richards and Richards, 1979).

Cuticular extensions include spine-like, bristle-like or hair-like features, as well as other processes originating from both the external and internal surfaces of the cuticle (Gorb, 2001; Vincent and Wegst, 2004). Many multifunctional micro/nanostructures are present on the insect cuticle surface, and these structures have a variety of properties (Hu *et al.*, 2011a; Hu, 2014).

The functions of these different morphological, functional and developmental protuberances are not well known (Richards and Richards, 1979; Gullan and Cranston, 2010). It is, however, known to have mechanical, self-cleaning, adhesive, optical, actuation, sensing and responsive functionalities (Hu, 2014) that enhances insect survival. Long, stiff spines are used for defence (Richards and Richards, 1979). The functions of trichoid sensilla are sensory, including touch, taste and smell (Richards and Richards, 1979). Tactile setae that include bristles or chaetae (a soft bristle made of chitin) are used for mechanical purposes, namely, to track or detect vibrations (Richard and Richard, 1979) or as tools for grooming (Gorb, 2001). They can also serve as a defensive mechanism, for example the barbed setae (hastate, pedicle - a small stalk-like structure) in dermestid beetle larvae (Richards and Richards, 1979). Some types of setae are also involved in dispensing pheromones (Richards and Richards, 1979; Gorb, 2001). The cuticular microstructures, acanthae, are spines or any sharply pointed projection structure that originate from one cell (Gorb, 2001). These setae are not moveable, since sockets are lacking (Richards and Richards, 1979).

Surface structures on cuticles also influence the surface properties in terms of wettability and surface adhesiveness (Schroeder *et al.,* 2018), as these micro- and nanostructures assist when in contact with water or other sticky substances (Hu, 2014). Cuticle surface topography is a useful parameter in the study of wettability of insect surfaces. Absorption from particles such as water or chemicals are delayed by structures that have pronounced hydrophobic, or in some cases, superhydrophobic surfaces (Hu, 2014). Cuticle roughness improves hydrophobicity, which results in cuticles with non-wetting surfaces, which enables insects to resist wetting and protects them from foreign substances, thereby ensuring survival under conditions of high humidity or flooding (Hu, 2014). Cuticle topography such as spikes, horns, bumps and pores allow air to occupy the surface of the cuticle, minimizing wetting by water droplets (Schroeder *et al.,* 2018). Cuticle topography with smaller spacing between protuberances allows for less wetting compared to those with larger spacing (Bhushan and Jung, 2008). Insect cuticles with various protuberances resist wetting of the underlying membranes better when in contact with water droplets, due to the air pockets formed underneath the water droplets (Schroeder *et al.,* 2018). The insect cuticle is superhydrophobic depending on the interaction between the roughness of the cuticle and the moving droplets, with water droplets bouncing off the surface rather than completely wetting it (Hu, 2014).

Adhesion to the surface structures that occur on insect cuticles keeps surfaces clean from droplet-based fluids (Blossey, 2003). Wettability and the contact area may affect adhesion and can inhibit and/or prohibit functionalities on the surfaces of insects, which indirectly influences their life expectancy (Burton and Bhushan, 2005).

Insecticides enter the body of an organism through contact, ingestion or respiration (Yu, 2003). Since the characteristics of insect cuticles affect the penetration into insecticides, it also influences the efficacy of insecticides applied for their control.

### 1.6 Application of insecticides and droplet entrapment

Insecticides are used to protect crops from insect pest damage, with the aim to reduce yield losses (Mathews, 2000). Effective insecticide application methods are therefore important (Mathews, 2000), followed by rapid action of the toxin on the pest insect (Casida, 2010). Since crop production is under constant pressure, applicators of pesticides should aim to handle and apply pesticides as effectively as possible by following label recommendations on dose, economic thresholds, and careful calibration of equipment (Hofman and Solseng, 2004). The position of the pest and its movement on crop plants should also be considered (Nansen and Ridsdill-Smith, 2013). This will determine whether the formulation must be delivered to a specific portion of the canopy and if the application must be completed within a certain time window (Nansen and Ridsdill-Smith, 2013). Characteristics of the target canopy also influence the spray deposit on the target (Hanafi *et al.,* 2016).

Spray coverage is affected by the volume of insecticide spray applications (Nansen and Ridsdill-Smith, 2013). The required insecticide application rate and information on the volume of the carrier (mostly water) are provided on insecticide labels (Nansen and Ridsdill-Smith, 2013). When using modern spray equipment, the application rate is controlled by three variables, namely speed of the vehicle, output of the spray nozzle, and the boom height above the canopy (Nansen and Ridsdill-Smith, 2013). The forward speed and spray nozzle spacing, as well as the appropriate nozzle type, determine whether pesticide distribution onto the target area is sufficient to provide the desired application rate (Hofman and Solseng, 2004). Effective pesticide application can therefore be achieved by selecting appropriate application equipment (Hanafi *et al*., 2016). The efficacy of the toxicants that are applied is, however, also significantly influenced by environmental factors (Pimentel and Levitan, 1986). Environmental factors impact on insecticide spray depositions and drift (Nansen and Ridsdill-Smith, 2013).

Droplet size affects insecticide deposition onto the target pest individuals (Hanafi *et al*., 2016), and therefore also affects its efficacy (Pimentel and Levitan, 1986). Droplets must provide sufficient coverage of the target (Yu et al., 2009), and provide control of the amount of pesticide reaching the target (Boina *et al.*, 2013). Pressure and the type of nozzle convert the spray mixture into droplets (Beyaz *et al.*, 2017). For example, smaller droplets are produced under higher pressures (Hanafi *et al*., 2016). To achieve effective control, droplets should be small enough, but because of evaporation during droplet transportation, droplets should also be large enough to reduce drift potential and to deposit onto the target (Yu e*t al.*, 2009). On the contrary, pesticide application with large droplets may reduce the control efficiency and lead to excessive pesticide use (Yu *et al.*, 2009). The desired droplet size for insecticides, therefore, ranges from small to medium size (Al-Sarar, 2003). Application of pesticides at the optimal droplet size is key to successful treatment and effective management of pests (Boina *et al.*, 2013).

Upon reaching the target, droplets spread and are influenced by surface structures of the target (Yu *et al.*, 2009). Droplet entrapment by cuticle surface roughness and micro-structures affects cuticle wettability and absorption that could cause less effective uptake of applied pesticides (Hu *et al.*, 2011a,b; 2014; Schroeder *et al.*, 2018). The efficiency of insecticide application and efficacy of the insecticide is therefore partly dependent on droplet size and surface characteristics of the target (Yu *et al.*, 2009).

Waxy surfaces negatively affect water-based pesticides (Tak and Isman, 2015). This barrier is overcome by adding wetting agents that reduce surface tension, allowing droplets to spread more evenly (Czarnota and Thomas, 2013). By altering the spreading abilities of applied liquid droplets through adding wetting agents, anti-wetting functionalities of cuticle surface structures could be affected.

A further consequence of insufficient deposits onto the nanostructures of the target pest cuticle, caused by poor insecticide spray application methods, is resistance evolution to insecticides (Al-Sarar *et al*., 2006). High levels of single-gene resistance evolution are promoted with overdosing, whereas low dosage exposure of active ingredients contributes to polygenic resistance (Al-Sarar *et al*., 2006).

## 1.7 Problem statement

Evolution of resistance can occur through several mechanisms, one of which is penetration resistance. Study of the deposit pattern of pesticide droplets on the surface of the target will result in a better understanding of the mechanism of penetration resistance, and could lead to development of improved application methods (Yu *et al.*, 2009). The characteristics of insect cuticles, such as cuticle thickness and entrapment of droplets on the cuticle surface and by the cuticle surface structures, can also affect application efficiency and penetration of insecticides. These aspects have not been studied before for the lepidopteran pests of maize in South Africa.

## 1.8 Objectives

### 1.8.1 Main objective

**The main objective was to determine the cuticle thickness of *S. frugiperda* and to observe surface structures as well as their potential role in droplet entrapment of *C. partellus, B. fusca*, *H. armigera* and *S. frugiperda.***

### 1.8.2 Specific objectives

To study and:

1. determine the cuticle thickness of third- to sixth-instar *S. frugiperda* larvae,
2. observe the cuticle surface appearance and external cuticle protuberances of third to sixth-instar *C. partellus, B. fusca, H. armigera* and *S. frugiperda* larvae.
3. study droplet entrapment of water and a water + wetting agent mixture by cuticle protuberances of third and sixth-instar larvae.

The results of this study are presented in the form of chapters with the following titles:

- Chapter 2: Cuticle thickness and surface protuberances of lepidopteran larvae
- Chapter 3: Droplet entrapment by cuticle protuberances of lepidopteran larvae
- Chapter 4: Conclusions and recommendations.

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# CHAPTER 2

# CUTICLE THICKNESS AND SURFACE PROTUBERANCES OF LEPIDOPTERAN LARVAE

## 2.1 Abstract

This study aimed to investigate the mesonotal cuticle thickness of *Spodoptera frugiperda* larvae from different instars and the cuticle protuberances of four lepidopteran species, *viz*. *S. frugiperda, Helicoverpa armigera, Chilo partellus* and *Busseola fusca.* Cuticle thickness and the distance between bases of major spikes of third- to sixth-instar *S. frugiperda* larvae were measured and micrographs of surface appearances were captured using a Scanning electron microscope (SEM). A significant difference in cuticle thickness between younger (third and fourth instar) and later instar (fifth- and sixth-instar) *S. frugiperda* larvae was demonstrated. A thickened cuticle layer can reduce the amount of chemicals that enters the body and delay insecticide penetration. The thickened cuticle may therefore contribute, together with other mechanisms, to older larvae being more tolerant to insecticides. The cuticular surface topography differed between species. The protuberances of *C. partellus, B. fusca* and *H. armigera* were mainly conical in shape, while those on the pronotum of *S. frugiperda* were dome-shaped. Dome-shaped cuticular structures enable elasticity, growth and elongation of the epicuticle. The processes on the cuticle of *H. armigera* are spines, horns, acanthae and microtrichia. Other microstructures, blister-like swellings, are also present on the cuticles of *S. frugiperda*, *C. partellus* and *B. fusca*. The waxy layer on the cuticles of all four lepidopteran species is an essential part of an insect cuticle to prevent water absorption and repel water from the surface.

**Key words:** cuticle; acanthae, penetration resistance, protuberances, spikes, wax layer

## 2.2 Introduction

The external surface of insects is covered with an extracellular layer, the cuticle (Anderson, 2009). The thickness of the cuticular layer varies, depending on the insect species, stages of development and body region (Anderson, 2009). Thickness typically ranges between 100 and 300 µm (Anderson, 2009). The cuticle of insects is not a flat sheet, but it is curved and corrugated (Vincent and Wegst, 2004). It has, amongst others, an important structural function, and forms sensors through which an insect engages with, and observes the outer world (Jan *et al.*, 2017; Balabanidou *et al.*, 2018; Eilenberg and van Loon, 2018). The structure of the cuticle is different in each species (Jan *et al.*, 2017) and it differs in surface sculpturing (Anderson, 2009). It serves various purposes (Nguyen *et al.*, 2014), for example to protect the insect from water loss, and it acts as a protective barrier to the outside (Vincent and Wegst, 2004; Jan *et al.*, 2017; Eilenberg and van Loon, 2018). The cuticula is covered by a wax layer that serves as the main waterproofing barrier, followed by a cement layer that protects the layer of wax on the outside (Vincent, 2001; Anderson, 2009). Environmental abrasion can remove the cement and wax layers, but it is renewed by epidermal cells, which secrete material in solution through the pore canals that extend through the cuticle (Vincent, 2001). The hydrophobic wax layer must to be overcome by topically applied pesticides to enter the hydrophilic environment of the endocuticle and the body cavity of an insect (Vincent, 2001).

Insects are well- equipped with cuticle extensions that are spine-like, bristle-like or hair-like, as well as with other processes from the external and internal surfaces (Gorb, 2001; Vincent and Wegst, 2004). These structures or outgrowths are called protuberances and can be classified into four fundamental types: (1) multicellular spines, which are cells similar in appearance to those of the general epidermis cells (Fig. 2.1a), (2) setae or trichoid sensilla (Fig. 2.1b), (3) unicellular or acanthae (Fig. 2.1c) and (4) subcellular or more than one projection per cell known as microtrichia (Fig. 2.1d) (Richards and Richards, 1979; Cranston, 2010). The exoskeleton is mostly covered with these protuberances, ranging from relatively flat scales to long hairs and brush-like structures with complexity in their geometry (Vincent and Wegst, 2004). The cuticular surface may, however, also be smooth, embossed or engraved (Richards and Richards, 1979).

Micro- and nano-structures are known to perform a range of functions (Hu *et al.*, 2011a). Diversity in the surface structures provides evidence that specific structures with different shapes and sizes are used for different functions by insects (Hu *et al.*, 2011b). Superhydrophobicity, direct wetting, low adhesion and self-cleaning are amongst these functions that improve survival of an insect in challenging environments (Gorb, 2001; Hu *et al.*, 2011a).

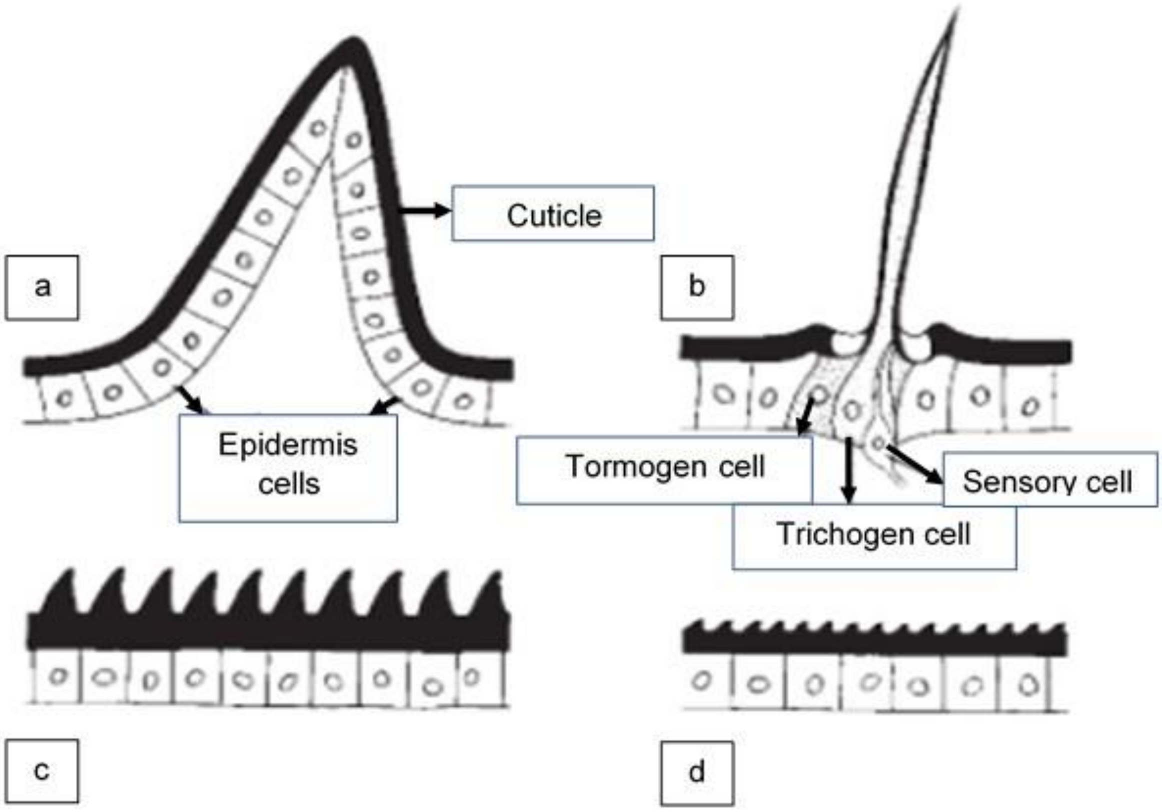

Figure 2.1 The four basic types of cuticular protuberances. a) A multicellular spine, b) a seta or trichoid sensillum, c) acanthae and d) microtrichia (From Cranston, 2010).

Droplet entrapment by cuticle surface roughness and micro-structures affects cuticle wettability and absorption that could cause less effective uptake of applied pesticides (Hu *et al.*, 2011a,b; Hu, 2014; Schroeder *et al.*, 2018). However, penetration resistance of insects to insecticides occurs when the outer cuticle of insects develops barriers that can slow the absorption or reduce the amount of chemicals that enter the body of an insect (Panini *et al*., 2016). For example, certain insecticide- resistant strains of insects may have a thickened cuticle layer, which could delay insecticide penetration (Ahmad *et al*., 2006).

Four important lepidopteran pests on maize in Africa are the Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Midega *et al*., 2018), the African bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), and the two stalk borers, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) (Botha *et al*., 2019). The aim of this study was to determine the cuticle thickness and to observe the cuticle surface appearance and external cuticle protuberances of third to sixth-instar larvae of these four species.

## 2.3 Material and methods

### 2.3.1 Lepidopteran species

Larvae were sampled from maize fields in Limpopo and North-West provinces, South Africa. *Spodoptera frugiperda* and *B. fusca* were collected at the Staankraal farm, Makoppa, Limpopo, (24˚26'S 27˚05'E)*, H. armigera,* at Ventersdorp, North West (26˚10'S 26˚67'E), *C. partellus* at Bloemhoff farm, Makoppa, Limpopo (24˚48'S 27˚12'E). The collected larvae were reared in plastic containers (360 mm (L) x 260 mm (B) x 150 mm (H) on maize leaf material until pupation.

Three Petri dish lids (±5 cm in diameter), containing a layer of small crusher stones, were placed in plastic containers (360L x 260B x 150H mm with a mesh lid) and water was added every second day, up to a three-quarter level of the height of the stones, to provide humidity. Fifteen pupae of a single species were placed in the bottom part of a Petri dish, on the crusher stones, per container. Folded wax paper and fresh maize leaves were provided as oviposition substrates. A 10% sugar solution was provided in a 50 ml plastic tube, sealed with cotton wool as food for the moths that emerged from the pupae. Containers were closed with a mesh-infused lid and maintained in a temperature-controlled room at 26 ± 1 ˚C and RH of 65 ± 5%. Maize leaves were replaced every third day until no more moths survived. Eggs were collected daily and transferred to small plastic containers with a mesh-infused lid (27 mm high and 17 mm in diameter). These containers were kept in a desiccator in which RH was maintained at 70 ± 5% using a potassium hydroxide solution according to the method of Solomon (1951). The eggs were observed daily until larvae hatched. After hatching, neonate larvae were transferred to containers with fresh plant material as a food.

*Spodoptera frugiperda* and *H. armigera* larvae were reared in mass from the first- to the third-instar in plastic containers (360L x 260B x 150H mm) and provided with fresh maize plant material as food. Fourth to sixth-instar larvae were reared individually on fresh plant material, in aerated plastic containers (52 mm high and 30 mm in diameter) to avoid cannibalism. When fresh leaf material was not available, these rearing colonies were provided with maize ears in the soft dough stage.

Neonate *C. partellus* and *B. fusca* larvae were reared in similar plastic containers to those described above. Larvae were reared on fresh maize whorl leaf tissue. From the fourth instar onwards, larvae were provided with maize stems as food, until pupation. Rearing was done in a rearing room, at 26 ± 1 ˚C, 65 ± 5% RH and 14L:10D photoperiod. Larvae from these rearing colonies were used for scanning electron microscopic (SEM) evaluations and to capture micrographs.

### 2.3.2. Preparation of larvae for microscopy

An indication of the positions of the thoracic plate, prothorax and mesothorax is provided in Figure 2.2.

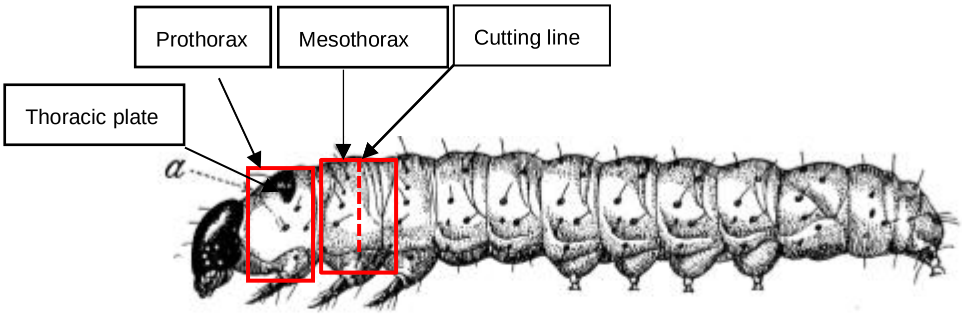

Figure 2.2 *Spodoptera frugiperda* larva with the pro- and mesothorax indicated. (From Luginbill, 1928). The cutting line is indicated as a dotted line, in the centre of the mesothorax.

Fresh larvae were killed wrapped in tissue and placed in the freezer for 10 minutes whereafter the thoraxes were cut. For ease of handling, the head and prothorax were immersed for >8 h (overnight) in Todd's fixative (Todd, 1986) at 4 ˚C (Fig. 2.3a). These samples were rinsed 3 times in 0.05M cacodylate buffer for 15 minutes after removal from the Todd's fixative and then immersed in 1% osmium tetroxide (in cacodylate buffer) for 1 h. The samples were then rinsed three times for 15 minutes in distilled water, followed by dehydration for 15 minutes in each concentration of the following ethanol series (50, 70, 90, 100 and 100%) (Fig. 2.3b). The specimens were critical point dried (Fig. 2.3c) and mounted onto aluminium stubs (Fig. 2.3d) with double-stick carbon tape (Fig. 2.3e) and sputter-coated with gold/palladium (Fig. 2.3f, g). The front part of the larvae (head and thorax) was prepared for SEM. The cuticular surface structures on the dorsal prothoracic area, behind the thoracic plate were studied and the distances between the bases of major spikes were measured. For measurement of the cuticle thickness, a cross section was made through the central part of the mesothorax (Fig 2.2). These areas were examined since insecticides are generally applied topically onto these areas in laboratory insecticide resistance studies (e.g., Kranthi, 2005; Durmuşoğlu *et al*., 2015). Examination was done by means of a FEI Quanta 200 Environmental Scanning Electron Microscope (ESEM). Multiple measurements of the cuticle thickness of the dorsal mesothoracic area of third-, fourth-, fifth- and sixth-instar *S. frugiperda* larvae were done. Measurements from 15 larvae per instar were done. Examination of measurements was done on the SEM micrographs and only exact measurements of the cuticles were included in the database for analyses. Cuticular surface structures were observed, and measurements of the distance between protuberances from the edge of their bases were done on a minimum of five larvae of each instar per species.

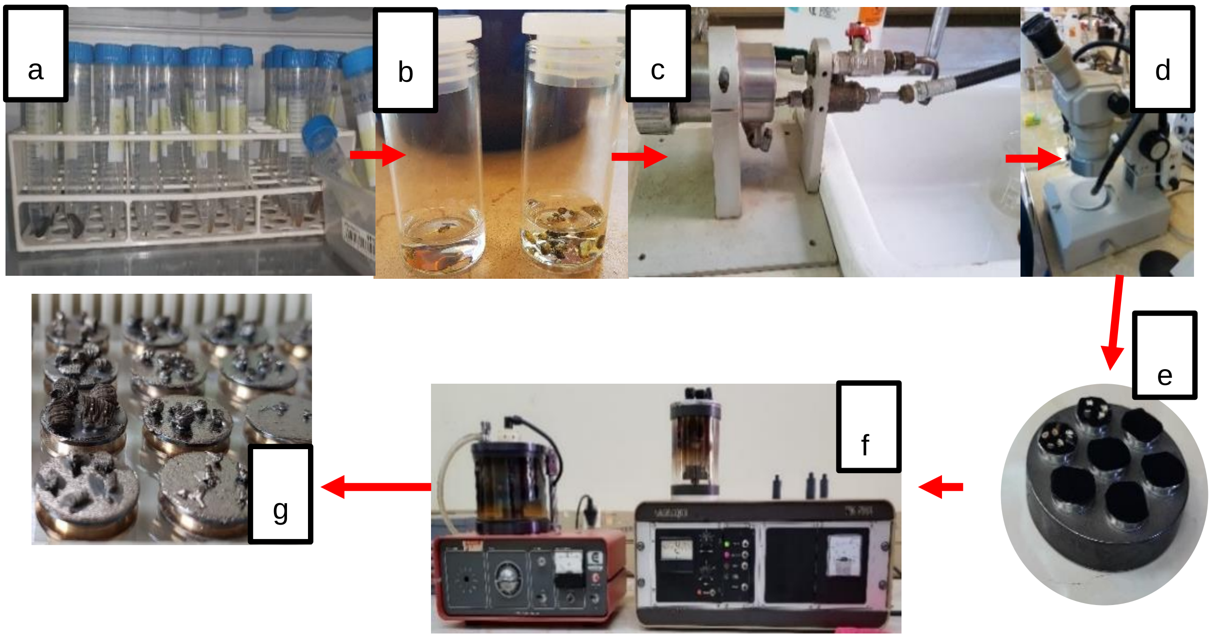

Figure 2.3 Preparation series for scanning electron microscopy (SEM). a) Larvae stored in tubes, b) samples immersed in a series of fluids, c) critical-point dryer, d) stereo microscope used to mount samples, e) samples before coating, f) gold/palladium Emscope TB500 used to coat samples, g) coated samples for SEM.

### 2.4 Statistical analysis

Measurements of the mesonotal cuticle thickness of *S. frugiperda* larvae, as well as data on the distances between the major spikes on the pronota cuticle surfaces of the larvae from the respective lepidopteran species, were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). The data were neither normally distributed, nor homogenous, and were therefore analysed by means of non-parametric Kruskall-Wallis tests, followed by Dunn's multiple comparison post hoc tests. The analyses were performed with TIBCO Statistica™ 13.3 (TIBCO Software, Inc., 2017).

## 2.5 Results

### 2.5.1 Cuticle thickness

Mean cuticle thickness of third- and fourth-instar *S. frugiperda* larvae was significantly thinner compared to those of fifth- and sixth-instar larvae, with no significant difference in cuticle thickness of third- and fourth-instar and between fifth- and sixth-instar larvae, respectively (Fig. 2.4). The cuticle thickness of the third- and fourth-instar larvae was < 220 nm (Figs. 2.5 and 2.6), while cuticle thickness of fifth- and sixth-instar larvae was >600 nm (Figs. 2.7 and 2.8).

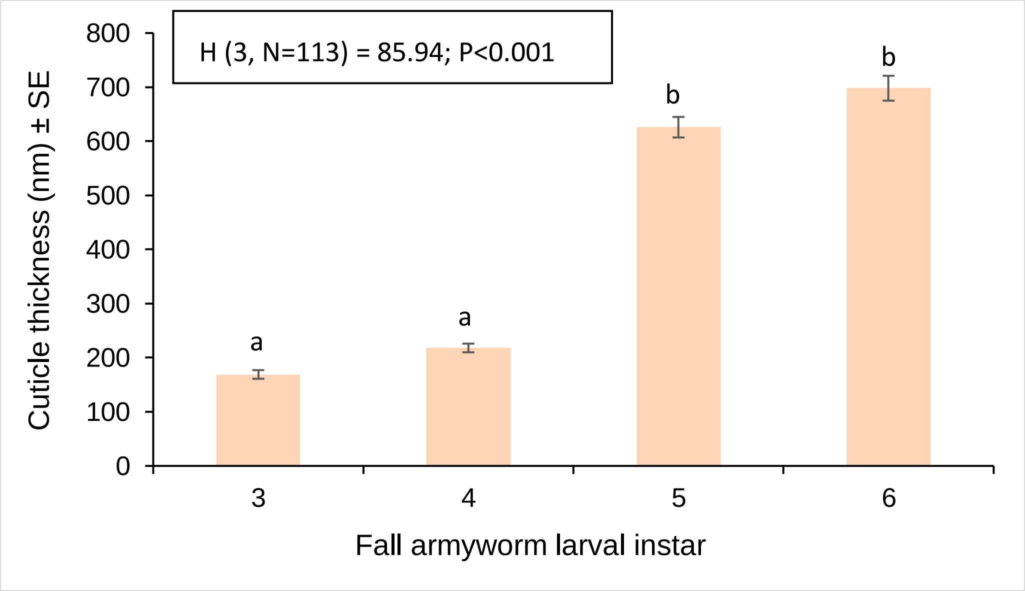

Figure 2.4 Mean cuticle thickness (±SD) of third to sixth-instar *Spodoptera frugiperda* larvae (Dunn's multiple comparison post hoc tests).

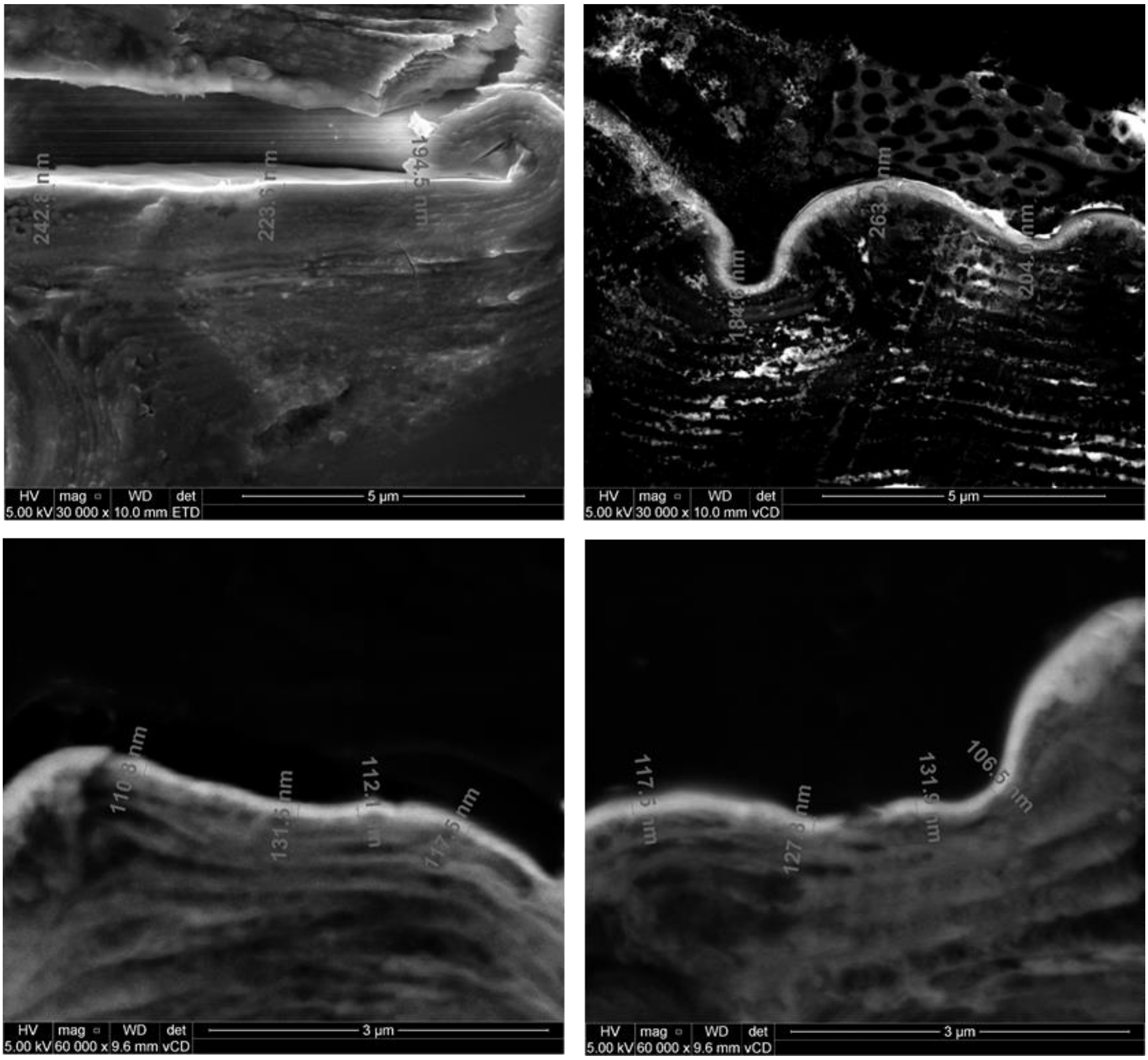

Figure 2.5 Cross section through the cuticle of third-instar *Spodoptera frugiperda* larvae, indicating measurements of the mesonotum width (cuticle thickness).

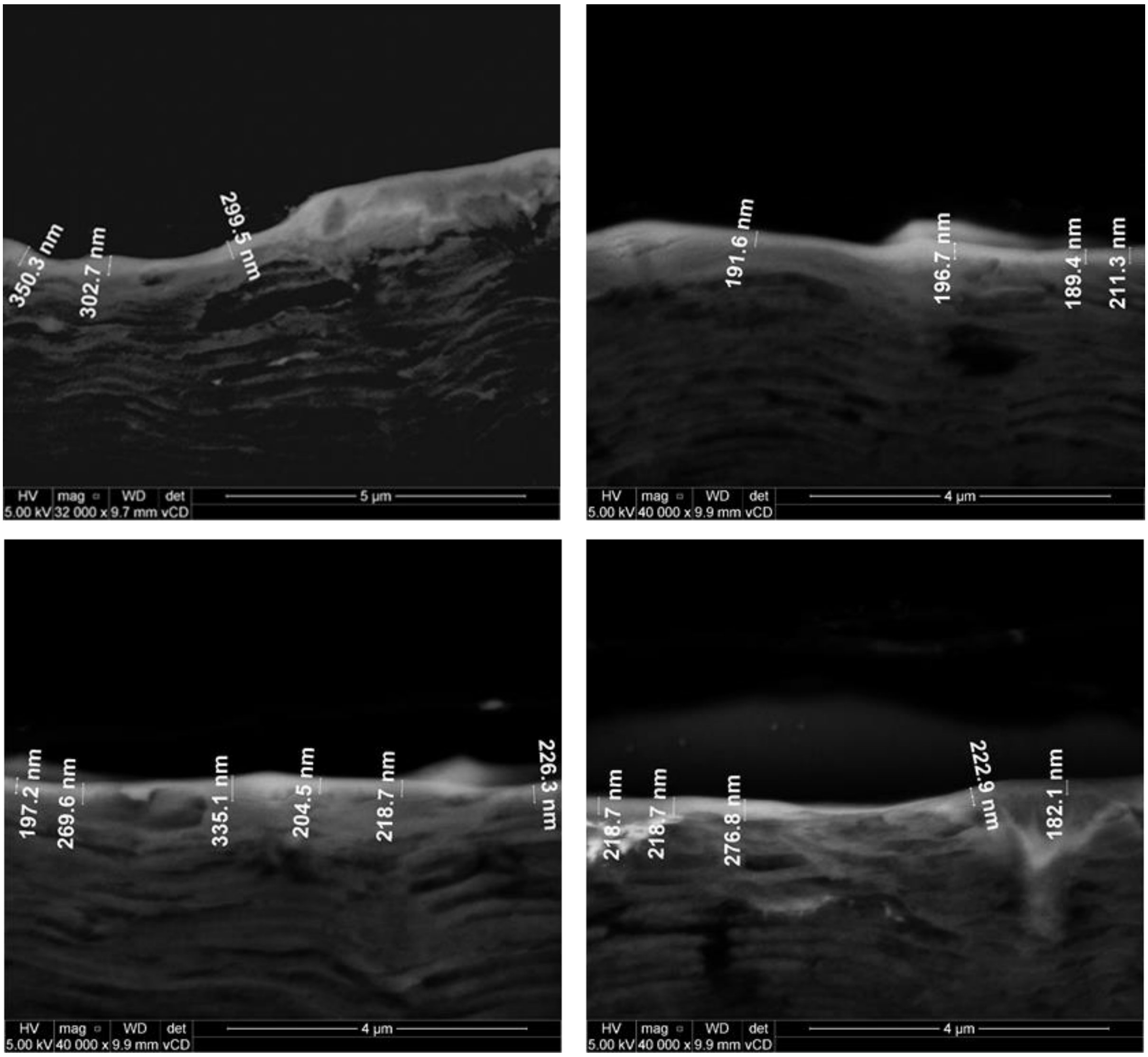

Figure 2.6 Cross section through the cuticle of fourth-instar *Spodoptera frugiperda* larvae, indicating measurements of the mesonotum width (cuticle thickness).

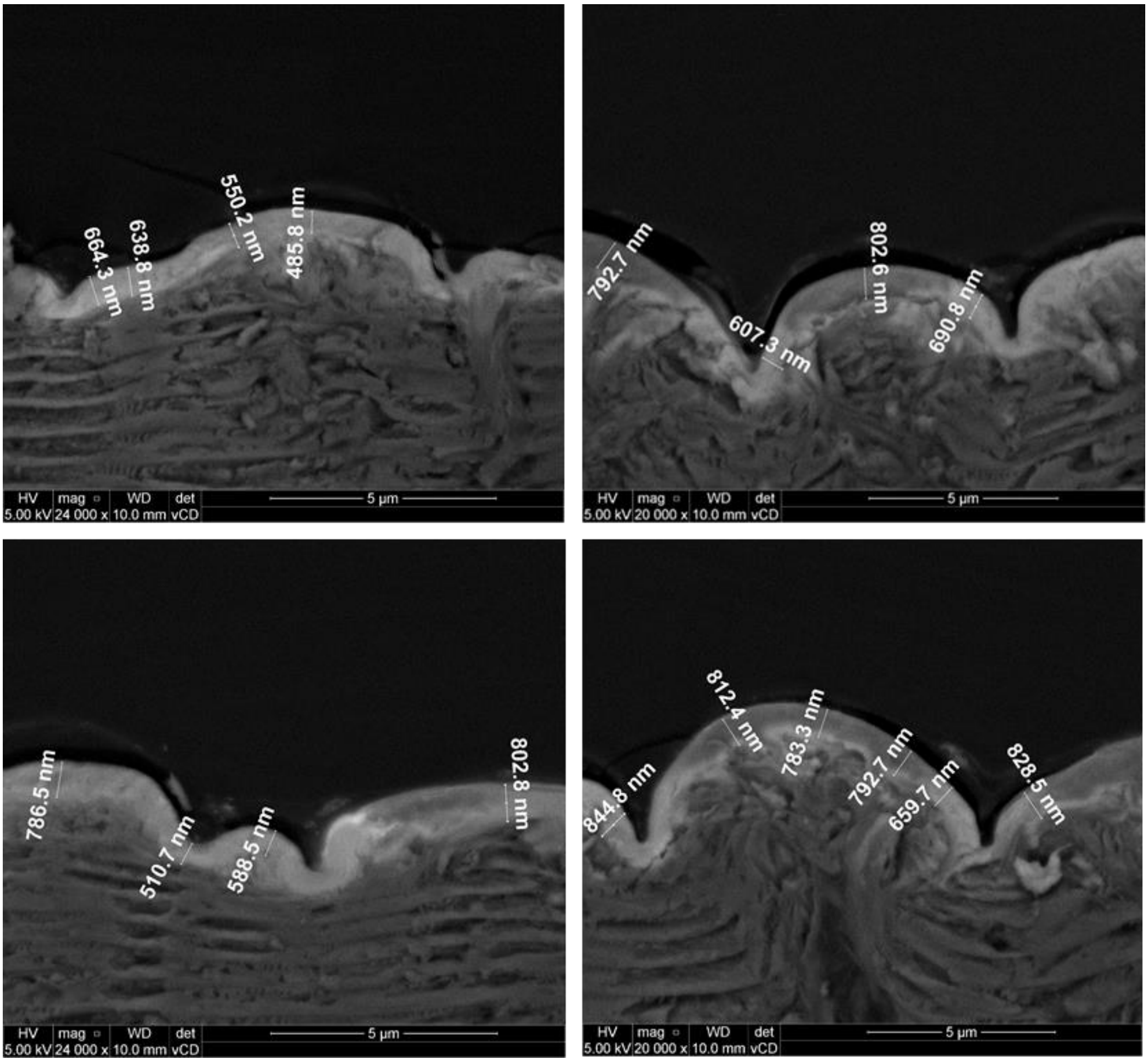

Figure 2.7 Cross section through the cuticle of fifth-instar *Spodoptera frugiperda* larvae, indicating measurements of the mesonotum width (cuticle thickness).

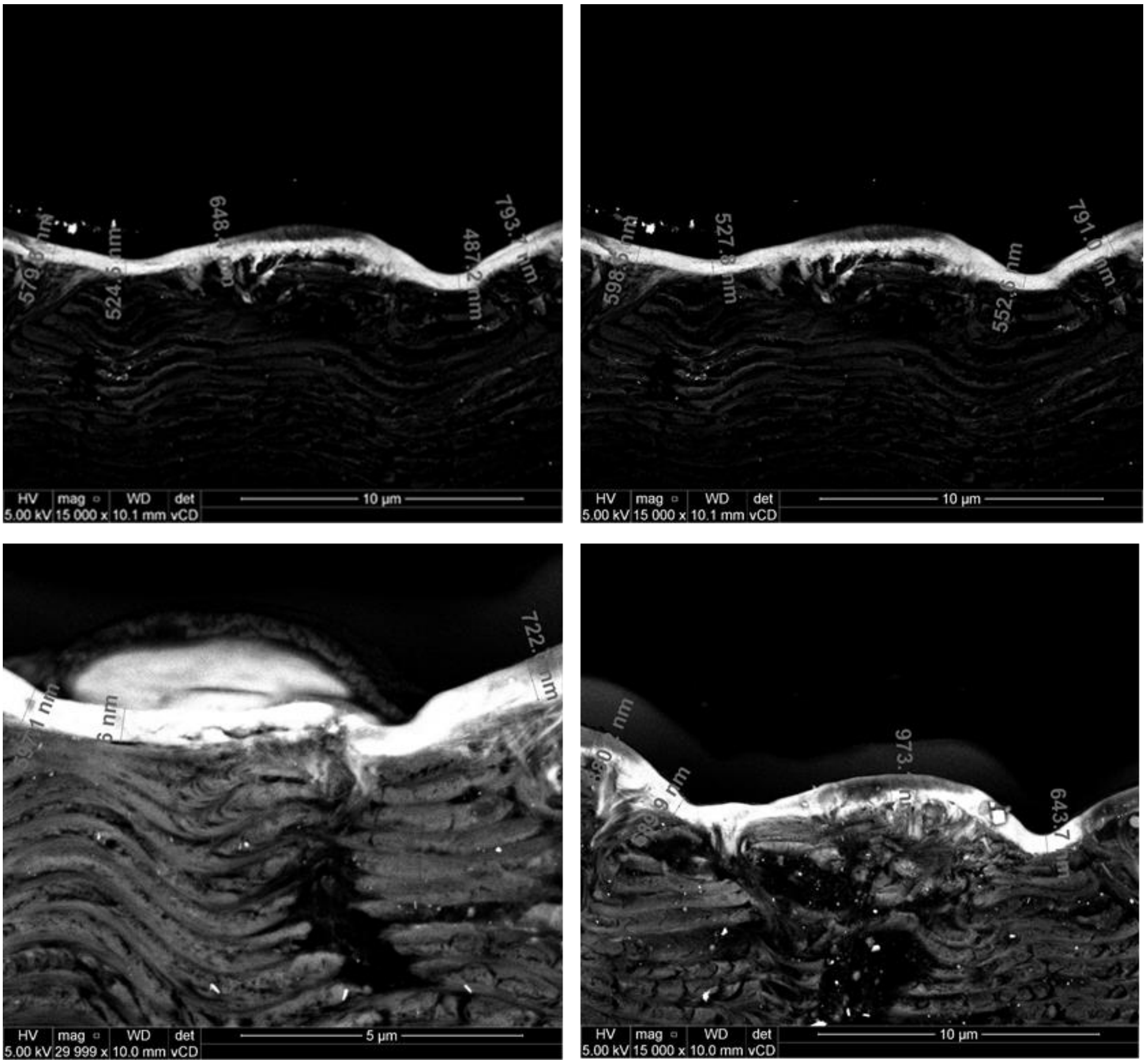

Figure 2.8 Cross section through the cuticle of sixth-instar *Spodoptera frugiperda* larvae, indicating measurements of the mesonotum width (cuticle thickness).

### 2.5.2 Cuticle surface appearances

The cuticle surface protuberances of *S. frugiperda* larvae from the third- to the sixth-instar on the pronota, consist of small to medium, curved or dome-shaped projections that are slightly pointed (Fig. 2.9a). The protuberances of the cuticle of *H. armigera* are medium and large, pointed conical protuberances (Fig. 2.9b). The closely arranged topography of the cuticulin is unique to this species when compared to the other species observed. Micro-organisms were observed between protuberances on the cuticle. Micro-organisms were also visible between acanthae, spines and horns on the cuticle surface of *C.*

*partellus.* Large quantities of wax are visible and the microtrichia and acanthae present on the cuticle surface of *B. fusca,* are slightly pointed. The surface topography of *C. partellus* (Fig. 2.9c) and *B. fusca* (Fig. 2.9d) is comparable, with both indicating periodic arrays of unicellular processes, the microtrichia and acanthae. The cuticle topography of *C. partellus* and *B. fusca* larvae from the third- to the sixth-instar was similar.

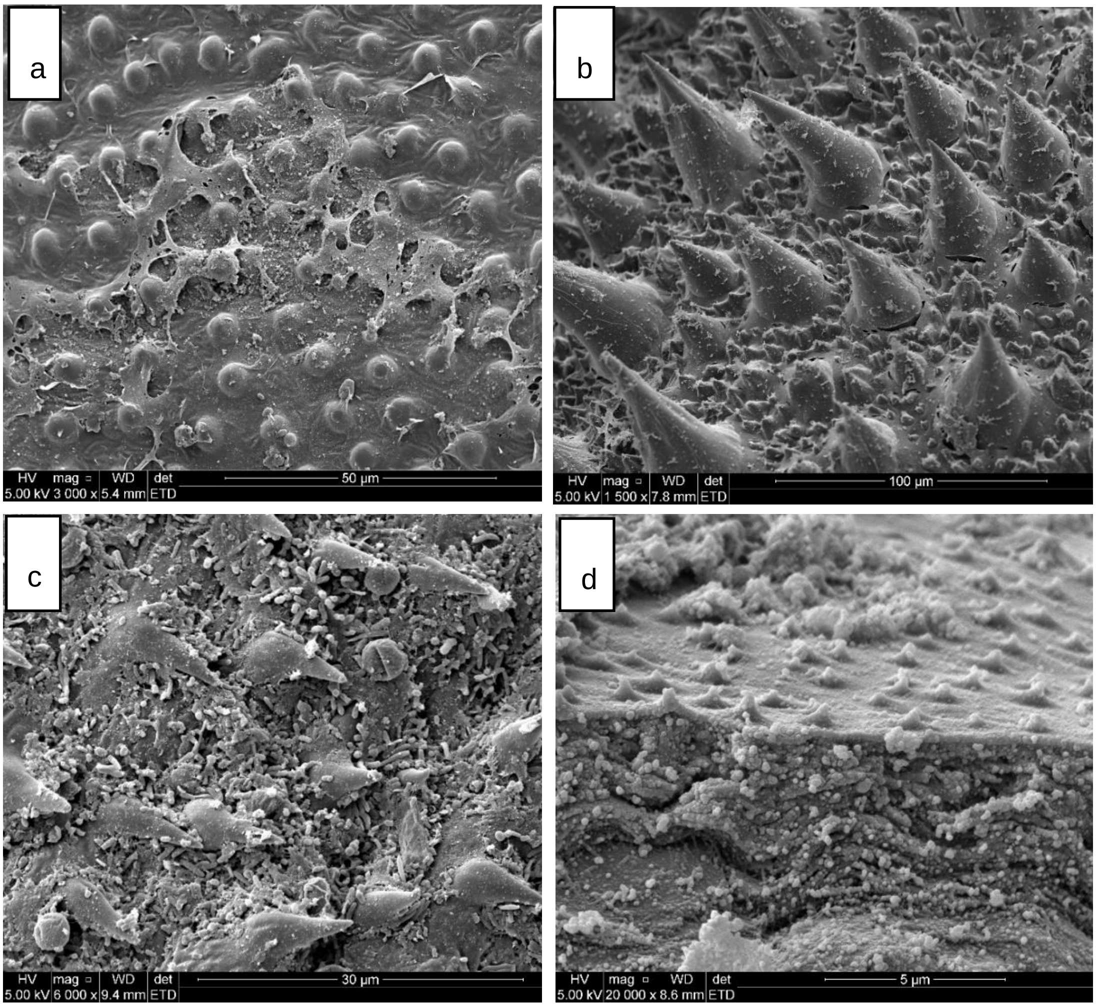

Figure 2.9 Micrographs depicting the typical cuticle surface topography on the pronota of a) *Spodoptera frugiperda* - top view of bumps with a wax layer present, b) *Helicoverpa armigera* - organized arrangement of different sized cone-shaped spikes, c) *Chilo partellus* - organized conical arrangement of spikes, d) *Busseola fusca* - small cone-shaped spikes.

### 2.5.3 Distance between protuberances

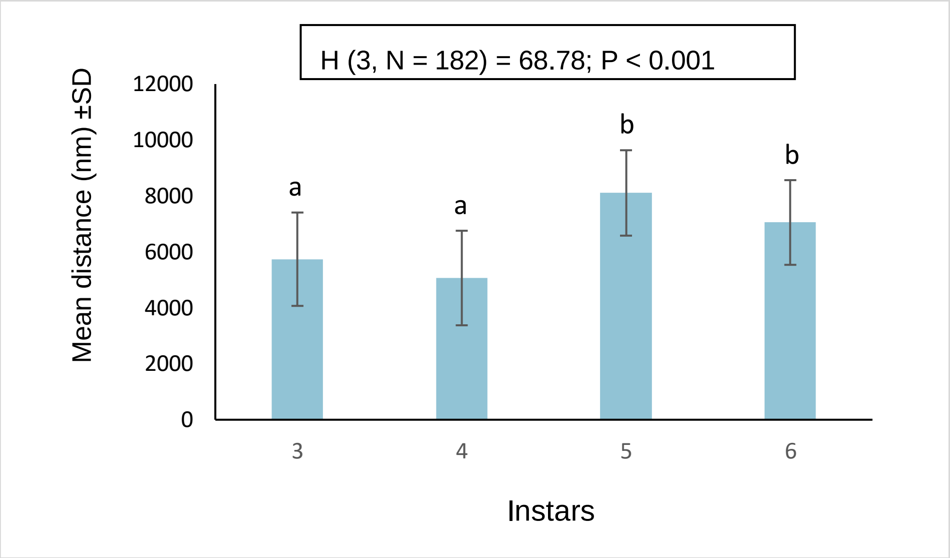

Figure 2.10 Mean distance (nm) (±SD) between surface protuberances on the prothorax of *Spodoptera frugiperda* larvae of different instars.

The mean distance between major spikes on the pronotum cuticle of third and fourth-instar *S. frugiperda* larvae did not differ significantly. There was, however, a significant difference between the mean distances of major cuticle spikes of the two smaller compared to the fifth and sixth-instar larvae (Fig. 2.10).

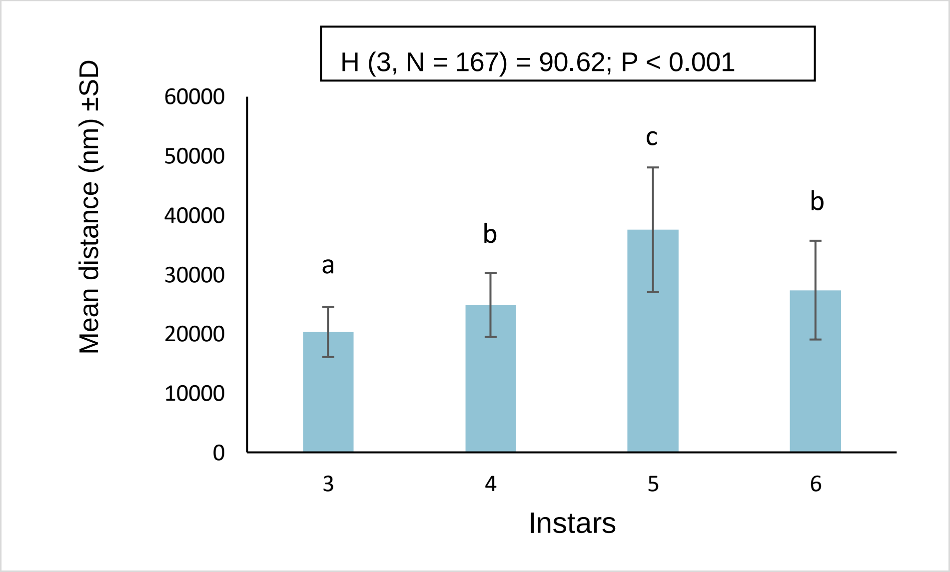

Figure 2.11 Mean distance (nm) (±SD) between surface protuberances on the prothorax of *Helicoverpa armigera* larvae of different instars.

The mean distance between major spikes on the pronotum cuticle of *H. armigera* larvae differed significantly between instars (Fig. 2.11). The spikes on the cuticle of fifth-instar larvae were the furthest apart, compared to the larvae from the other instars.

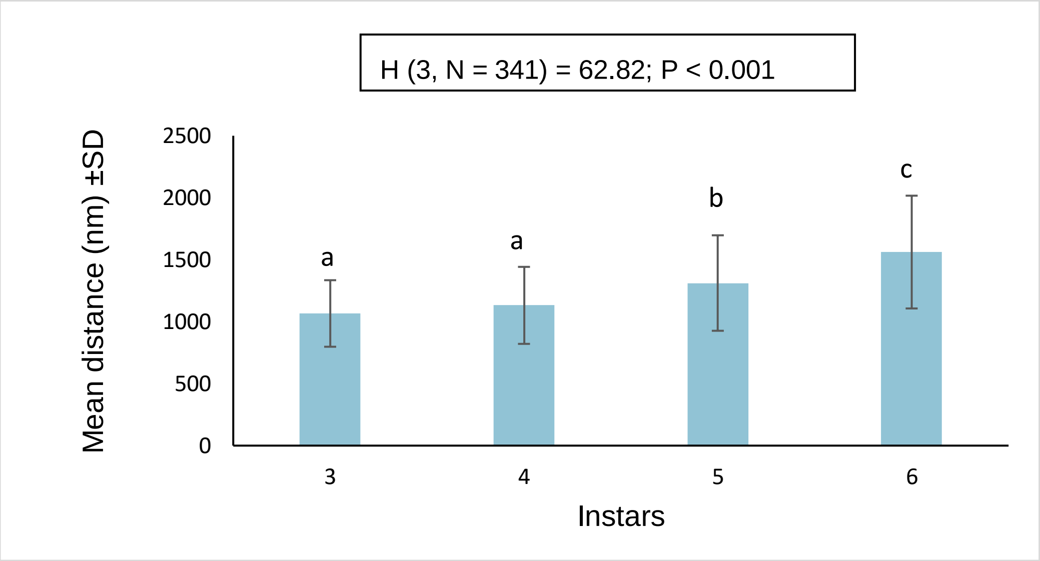

Figure 2.12 Mean distance (nm) (±SD) between surface protuberances on the prothorax of *Busseola fusca* larvae of different instars.

The mean distance between major spikes on the pronotum cuticle of third and fourth-instar *B. fusca* larvae did not differ significantly. The distances between major spikes on pronota of fifth- and sixth-instar larvae were significantly further apart, compared to larvae from the two smaller instars (Fig. 2.12).

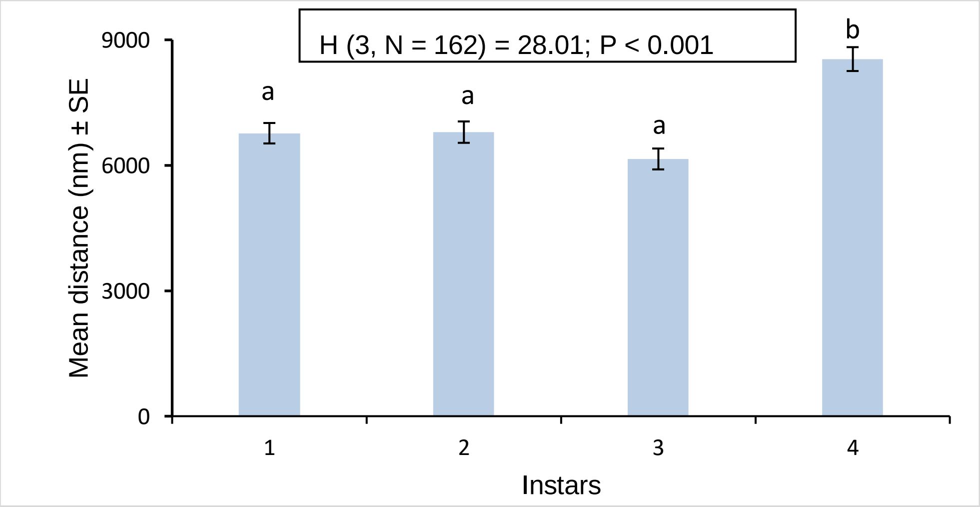

Figure 2.13 Mean distance (nm) (±SD) between surface protuberances on the prothorax of *Chilo partellus* larvae of different instars.

The mean distance between major spikes on the pronotum cuticle of third, fourth- and fifth-instar *C. partellus* larvae did not differ significantly. Distances between major spikes on the pronota of sixth-instar larvae were, however, significantly further apart compared to the smaller instar larvae (Fig. 2.13).

## 2.4 Discussion

### 2.4.1 Cuticle thickness

The cuticles of later instar *S. frugiperda* larvae (fifth- and sixth-instar) are significantly thicker than the cuticles of third- and fourth-instar larvae. The thicker cuticle of later instar larvae may partly be explained by moulting. The increase in larval surface area after moulting takes place through growth of the cuticle, both in terms of surface area and thickness (Williams, 1980). The epi- and exocuticle is shed during moulting, but the new cuticle becomes thicker in the following hours (Chapman, 1998).

Penetration resistance, where a toxin is absorbed more slowly into an insect's body, occurs when the outer cuticle develops barriers (Karaagac, 2012). A thickened cuticle may, amongst other factors, also contribute to lower permeability. Compared to third-instar *S. frugiperda* larvae, slower absorption of insecticides applied at the same dosage rate may occur in the later-instar larvae. It may therefore also contribute to the increased tolerance of late-instar larvae to contact insecticides.

Labels of insecticides registered for control of *S. frugiperda* in South Africa indicate that variable control can be expected if these insecticides are applied to late-instar larvae. This is not surprising, since it is known that insecticide tolerance of *S. frugiperda* larvae increases with each increasing instar (Yu, 1983; Mink and Luttrell, 1989).

The high levels of resistance of *Anopheles funestus* (G.M.J Giles) (Diptera: Culicidae) to permethrin was ascribed by Wood *et al*. (2010) to a thicker cuticle that absorbed insecticides slower, resulting in insecticides reaching the internal organs at a slower rate (Samal and Kumar, 2020). The decrease in insecticide susceptibility in the older *S. frugiperda* larvae, was ascribed by Yu (1983) to an increase in the metabolism of the insecticides in these larvae. This was also reported for deltamethrin resistance in *H.*

*armigera,* which was ascribed to poor permeability, as well as a high rate of deltamethrin metabolism inside the cuticle (Kumari *et al*., 2006).

### 2.4.2 Microstructures on the cuticle surface

Scanning electron microscope images of third to sixth-instar larvae showed a variety of protuberances on the cuticles of the respective species. The protuberances of *C. partellus, B. fusca* and *H. armigera* were mainly conical in shape, while those on the pronotum of *S. frugiperda* were dome-shaped. Uniformly shaped structures, such as the dome-shaped protuberance on the cuticle of *S. frugiperda,* can be important for antiwetting and self-cleaning (Watson *et al.,* 2017). The functionalities of setae and conical structures such as on the cuticles of *S. frugiperda*, *H. armigera*, *C. partellus* and *B. fusca* are described by Watson *et al*. (2017) to assist an insect in its interactions with a liquid (water). Nanoscale domes have antibacterial properties and aid in low adhesiveness (Watson *et al*., 2017). Unsal (2018) studied the integument of sixth-instar larva of *Galleria mellonella* (Lepidoptera: Pyralidae), and showed that the dome-shaped epicuticle was formed, similar to the waxy layer, by secretion of cuticular fluid from the epidermis through pores. A study by Hu (2014) reported the function of the arrangement of bumps, as a chain of stabilizing elements, designed to manage loading forces. Similar epicuticular tubercles observed as dome-shaped structures on *S. frugiperda* larvae, were reported by Unsal (2018) for *G. mellonella*, by Noble (1963) for *Podura aquatica* (L.) (Poduromorpha: Poduridae), and by Way (1950) for *Diataraxia oleracea* Hubner (Lepidoptera: Noctuidae) on larval integuments. These tubercles are characteristic of lepidopteran larvae (Way, 1950) and occur only in organisms with soft cuticles (Unsal, 2018). Dome-shaped cuticular structures enable elasticity, growth and elongation of the epicuticle (Unsal, 2018).

The processes on the cuticle of *H. armigera* are spines, horns, acanthae and microtrichia. Multi-functions of cuticle structures are common in insect species (Hu, 2014). Spines and horns refer to single protuberances that originate from cells similar in appearance to cells to the remainder of the epidermis (Gorb, 2001). Long, stiff, sclerotized cuticular spines are often used as defence mechanisms against enemies and predators (Gorb, 2001). Spines are also used for grooming, an important activity for insects in extremely dirty environments, since their sensory organs should be kept clean to ensure a response to external signals (Gorb, 2001). Acanthae are commonly known as unicellular processes or a single sclerotized protuberance, develop from a single cell, the trichogen cell (Gorb, 2001). Richards and Richards (1979) suggested that the derivation of this cell occurred from a trichoid complex, with the loss of both the tormogen and sensory cells, but it is not yet been proven (Gorb, 2001). In a study on the cuticle surfaces of sawfly larvae (Boeve *et al*., 2004), the microstructures, similar to those of *S. frugiperda*, *C. partellus* and *B. fusca,* were referred to as blister-like swellings, and to those similar to the microstructures on *H. armigera* larvae, as setae.

The droplet-shaped protuberances, also present on the cuticles of *H. armigera* larvae, and which end in slim, pointed processes were described by Fanger and Noumann (2001) to be responsible for a shagreen-like (rough granulated) topography of a cuticle. These structures are, however, so small (<100nm) (Watson *et al.* 2017), that they can only be observed using a high magnification microscope, such as the SEM. The biofilm of micro-organisms, visible on the cuticular spines of *H. armigera*, could possibly be ascribed to the dirty environment inside the rearing container.

A waxy layer was present on the cuticles of all four lepidopteran species studied. The typical thickness of the epicuticular wax layer in insects is 250 µm (Quéré, 2008). For example, it can be as thin as 1 µm in the hindgut and over gills of ephemeropteran larvae (mayflies), and thicker than 200 µm on the elytra of large beetles. This layer prevents water loss from an insect (Quéré, 2008), but also plays an important role in repelling water from the surface (Xu *et al.*, 2010), preventing it to entering the insect's body. Attachment of foreign bodies is generally reduced where waxy surfaces are present (Cribb *et al.*, 2010). The surface wax layer is also responsible for the darkening of an insect in humid environments, due to filaments that are incorporated into it (Filshie, 1982).

### 2.4.3 Contribution to hydrophobicity on the integument

The diversity of surface structures is used by the insect itself for either hydrophilicity or (super) hydrophobicity. Liquid spreads spontaneously on a hydrophilic surface, hence complete wetting occurs, while droplets remain in spherical form with no contact to the underlying cuticle on a hydrophobic surface (Quéré, 2008). Coexistence of roughness and structures such as setae contributes significantly to hydrophobicity (Quéré, 2008). Rough surfaces in combination with an organized arrangement of setae indicate a hydrophobic interaction with liquid droplets (Watson *et al.*, 2008).

Protuberances of the cuticle, which are frictional, are 0.5-30.0 µm long (Gorb, 2001). Results from this study indicated the distance between protuberances on the pronota of all four species to generally increase from younger to older instar larvae. The distance between protuberances can determine the volume of air trapped between the setae (Neumann and Woermann, 2009). The volume of air between adjacent spikes may facilitate anti-wetting of cuticle surfaces. Since the mean distance between protuberances on the younger instar larvae were shorter compared to larvae from later instars, it can be argued that more air per surface area can be trapped on smaller larvae that can contribute to better protection in terms of anti-wetting. This will aid in better protection of these larvae against liquid-based fluids in their environment.

## 2.5 Conclusion

The topography of the four lepidopteran species studied, *viz*. *S. frugiperda, H. armigera, B. fusca and C. partellus* differed. The protuberances on the pronota cuticles will, however, all contribute to anti-wetting of the larvae, which may necissate addition of an adjuvant to insecticides for effective control of these pests. A significant difference in cuticle thickness between younger (third- and fourth-instar) and later instar (fifth- and sixth-instar) *S. frugiperda* larvae was demonstrated in this study. It has an important application in chemical control of this pest and can be used as an explanation for application recommendations on insecticide labels with a contact action, registered for control of this pest. A thicker cuticle can, together with increased levels of detoxifying enzymes in older larvae, contribute to tolerance of these larvae to insecticides.

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## CHAPTER 3

# DROPLET ENTRAPMENT BY CUTICLE PROTUBERANCES ON THE CUTICLES OF LEPIDOPTERAN LARVAE

### 3.1 Abstract

Wetting of insect cuticles is important for effective control with spray applications of insecticides. Wettability is affected by the chemistry of the cuticle surface, as well as protuberances on the cuticle. Wax-coated spikes and/or bumps alters the hydrophobicity or hydrophilicity of the cuticle and allows non-waxy structures to efficiently capture water droplets. This study aimed to observe the differences in wettability of third- and sixth-instar larvae of lepidopteran maize pests, *viz*. *Spodoptera frugiperda*, *Helicoverpa armigera*, *Chilo partellus* and *Busseola fusca*. These larvae were sprayed with either water only, or water to which a non-ionic wetting agent was added. Stereomicroscope images showed that droplets were entrapped by the roughness of the cuticle of the respective species, as well as by setae on the cuticle surface. Surface hydrophobicity was reduced when a wetting agent was added to the water spray. Efficient application onto target pests requires specific droplet sizes provided by different types of nozzles. Applying insecticides according to the recommendations on the label, such as using the appropriate nozzle, spray volumes and spraying under the recommended conditions, improves wetting of an insect cuticle and results in better control of the target pest.

**Key words:** Droplet size, micro- and nanostructures, non-ionic wetting agent, setae maps hydrophobicity

## 3.2 Introduction

Maize (*Zea mays* L.) is one of the most valued crops in terms of food security and the economic value of the crop, for commercial as well as subsistence farmers in Africa (Midega *et al.*, 2018). Agricultural production and food security are negatively affected by the invasion of a diversity of lepidopteran pests (Midega *et al.*, 2018). The Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), an important pest of maize invaded Africa early in 2016 (Midega *et al.*, 2018). Other important lepidopteran pests of maize in Africa include *Busseola fusca* (Fuller) (Noctuidae), *Chilo partellus* Swinhoe (Crambidae) (Midega *et al.*, 2018) and *Helicoverpa armigera* (Hübner) (Noctuidae) (Kim *et al.*, 2018). The use of insecticides for control of these lepidopteran pests is often reported to be ineffective (Midega *et al.*, 2018). Efficacy of insecticides is, however, also affected by the application methods (Marini *et al*., 2015). When applications are done, care should be taken that droplets are homogenous in size to reduce drift and to ensure that they are deposited on the target areas (Bayat *et al*., 2011). Application is affected by nozzle type, droplet size and pressure, amongst other criteria (Nuyttens *et al.*, 2007).

The cuticle of terrestrial insects is highly hydrophobic (repelling water), which is an advantage in terms of their successful adaptation to their environment (Hischen *et al.*, 2017). It can, however, also be hydrophilic for some insects, allowing the surface or surface structures to interact with water, which then spreads over large areas (Hu, 2014) (Fig. 3.1). Both types of surfaces are considered to contribute to the natural self-cleaning function of insects (Hu, 2014). Self-cleaning is important for insects in environments where they get dirty or are exposed to unknown materials (Hu, 2014). To maintain mobility and functional efficiency, insects perform self-preening by removing or reducing surface contamination.

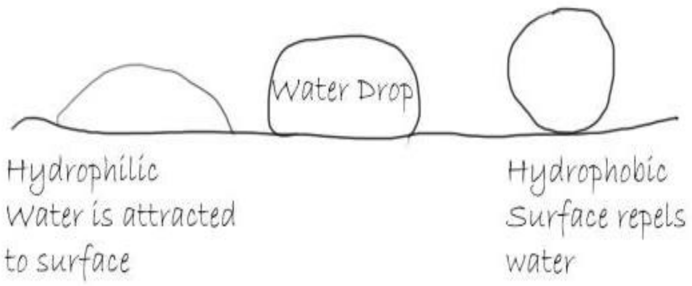

Figure 3.1. The interaction of hydrophilic and hydrophobic surfaces with water droplets. (https://askentomologists.com/2016/03/24/do-insects-get-trapped-in-water-drops-why-arent-they-constantly-drowning/

Structures on the cuticle of insects have developed through evolution and contribute to their survival and/or adaptation (Gorb, 2001). These structures on the external surface perform multiple functions that are important to their survival (Byun *et al.*, 2009). Wax-coated spikes and/or bumps alter the hydrophobicity or hydrophilicity and allow non-waxy structures to efficiently capture water droplets (Byun *et al.,* 2009). Water loss to the environment is restricted by the wax layer, which also prevents desiccation of the insect and contributes to super-hydrophobicity and self-cleaning (Mitov *et al.*, 2018).

Super-hydrophobicity is, amongst other factors, related to the topography of the surface. The interaction of water droplets with micro- and nano-structures on the cuticle can be described by either the Wenzel state, which describes a rough surface, completely wetted by a liquid droplet such as water, or by the Cassie-Baxter state, which is when a liquid droplet is prevented from wetting the contact area by being suspended on top of a rough surface, allowing air pockets in the topography (Byun *et al.,* 2009). Cuticle roughness/structures such as spines, horns (cones) and/or bumps (Fig. 3.2) can either uphold or trap droplets and act as significant physical barriers to the movement of droplets across the insect’s surface (Hu, 2014).

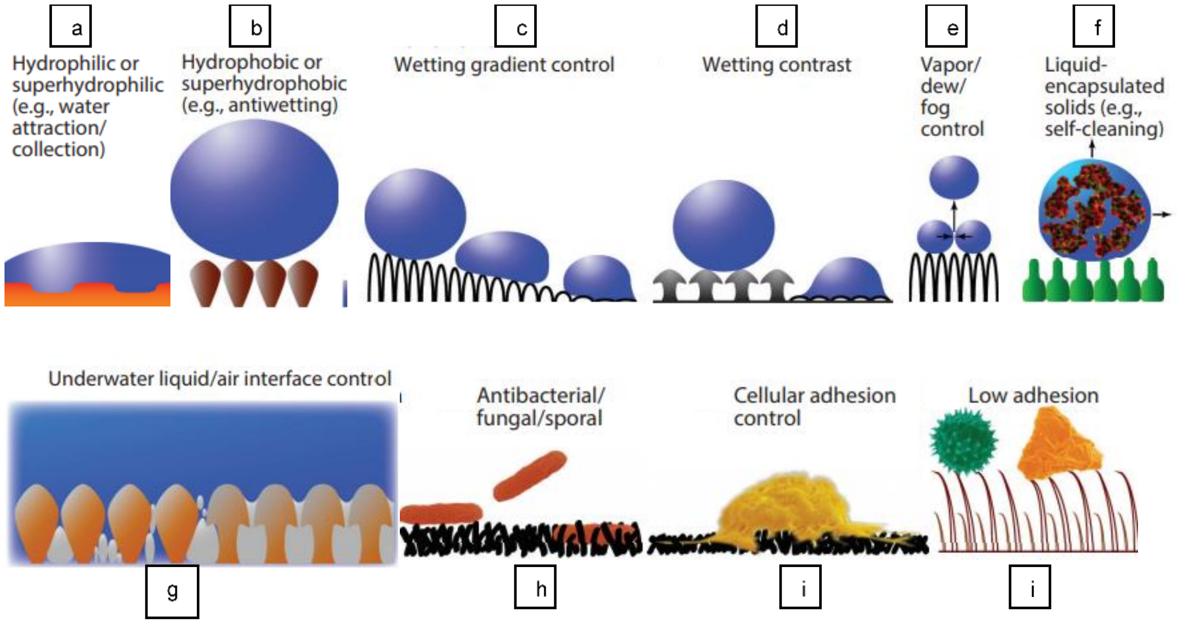

Figure 3.2 Functions of cuticle protuberances of insects depicted: a-g) interaction of liquids with various micro- and nanostructures on the cuticle; h) nanoscale domes or rods aid in antibacterial and low adhesive functions, i) structures with cellular – and j) a low adhesion function (From Watson *et al*., 2017).

Surface structures can be useful on surfaces requiring self-cleaning and waterproofing. Maintaining super-hydrophobicity requires stability at the complex solid-air-liquid interface (Byun *et al.,* 2009). Low adhesive surfaces and surface structures/roughness (Fig. 3.3) cause solid particles to roll off and/or restrict water droplets from entering the small spaces between the cuticle surface structures (Hu, 2014). The morphology of cuticles of different species therefore affects the formation of droplets and repellency of their surfaces (Xu *et al.*, 2010).

Protuberances on the hydrophobic surfaces of an insect cuticle reduce the contact area between the surface and water droplets or foreign substances and therefore limits adhesion (Hu, 2014). The effect of roughness on a water droplet to overcome the energy barrier from one state to another takes place when asperities are filled by the liquid, resulting in a more stable homogeneous interface (Fig. 3.3) (Byun *et al.,* 2009). Protuberances in the form of small clusters can serve as an anti-wetting protection layer (Hu, 2014). Although roughness promotes hydrophobicity, large flat, rough hydrophilic surfaces could also increase the wettable area that promotes water adsorption efficiency

(Hu, 2014). The topography and density of structures on cuticle surfaces are therefore important in anti-wetting (Byun *et al.,* 2009).

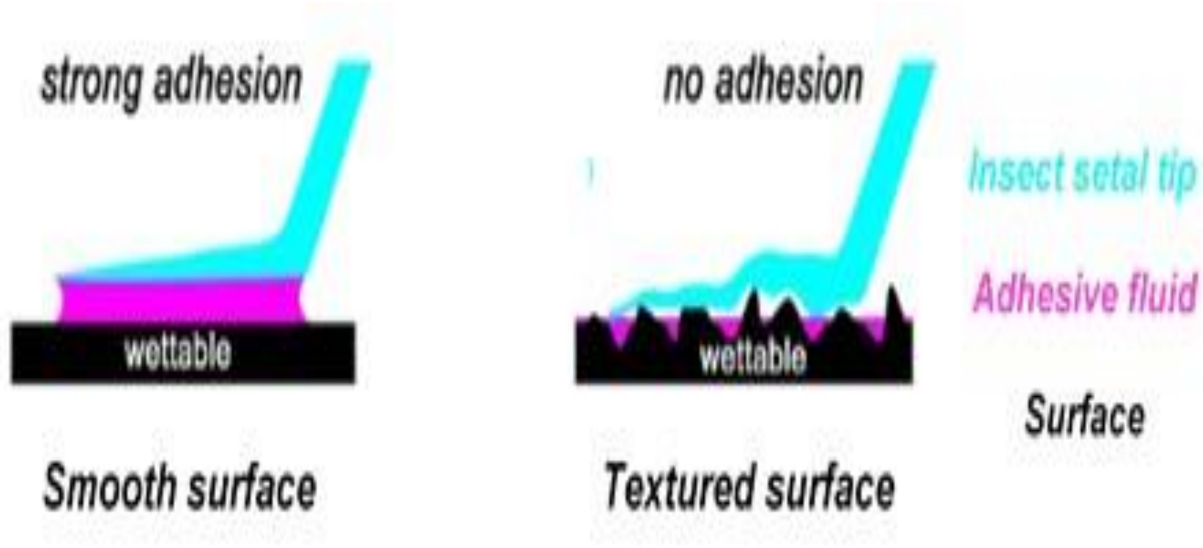

Figure 3.3 Wetting of an insect cuticle is affected by the roughness of the cuticle surface (From England *et al.*, 2016).

Insects covering themselves with small components from their surrounding environments as a defence mechanism rely on the adhesive properties of their physical micro- and nano-structures (Schroeder *et al.,* 2018). Studies on lacewings, termites and the water strider showed that setae keep droplets on the tips, preventing the underlying cuticle membrane from wetting (Watson *et al.*, 2010a,b). Hair compacted on the insect's surface also act as a layer of micro-springs that prevent penetration and/or dispersal of droplets at the contact area (Schroeder *et al.,* 2018). However, setae with grooves enhance stiffness that ultimately promotes direct wetting and reduces hydrophobicity on surfaces (Hu *et al.*, 2011).

Droplets can be repelled off the surface by large air pockets formed at the attachment region of setae or surface structures that are surrounded by small hollow regions or pores (Watson *et al*., 2011). Insecticide applications rely on the wetting properties of the cuticle for successful management of insect pests. Cuticle surface structures may, however, play a fundamental role in the absorption of the pesticide droplets.

Research to improve the efficacy of pesticide spray applications focuses mostly on methods and equipment to improve the accuracy of applications in reaching the target (Xu *et al.*, 2010). However, few studies have been done on droplet behaviour after it has been deposited onto targets. Knowledge about droplet behaviour on insect surfaces may contribute to effective control of insect pests. Understanding the wetting ability and/or droplet entrapment on target areas such as the cuticle surface and surface structures is important for the process of deposition, spreading of insecticides and penetration through the surface (Xu *et al.*, 2011).

Application methods are important for efficient dispersal of droplets. Aspects that should be taken into account include droplet sizes, environmental factors, timing of application and addition of a wetting agent (Matthews, 2008). During the application of sprays, droplets of varying sizes are deposited (Matthews, 2008). Nozzle characteristics determine these droplet sizes and therefore affect the spray quality and influence the interactions at the target area (Matthews, 2008).

Effective spreading of insecticides on a target organism and delayed absorption are achieved by including wetting agents to spray mixtures (Tu and Randall, 2003). Wetting agents are designed to improves spreading, dispersal and wetting, which reduces spray application problems and improve pesticide effectiveness. Wetting agents physically change the surface tension of a water droplet (Fig. 3.4), and are particularly effective on waxy surfaces or surfaces covered with setae and other structures to attain even spreading of the fluid (Czarnota and Thomas, 2013).

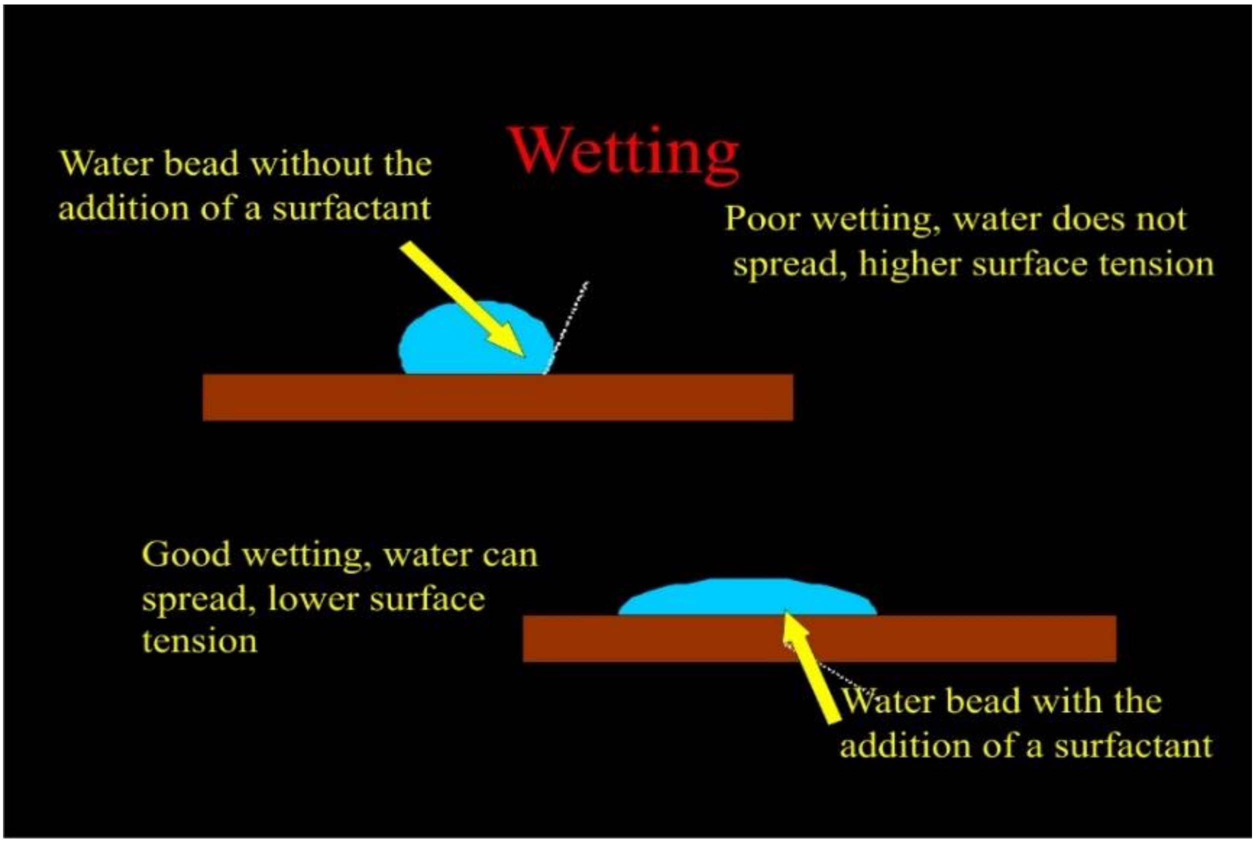

Figure 3.4 The effect of surfactants on the wetting properties of a water droplet. Addition of a surfactant reduces surface tension and promotes wetting of surfaces (From Czarnota and Thomas, 2013).

The effect of the cuticle protuberances of *S. frugiperda, H. armigera, C. partellus* and *B. fusca* on entrapment of water droplets has not been studied before. The aim of this study was therefore to investigate the effect of cuticle protuberances on the bodies of larvae of these four lepidopteran species on the entrapment of water droplets with and without a wetting agent added.

### 3.3 Materials and methods

For each species, *viz. S. frugiperda*, *H. armigera*, *B. fusca* and *C. partellus*, five third- and five sixth-instar larvae, randomly selected, were collected from the respective rearing colonies (See chapter 2). These larvae were placed between tissue paper to protect surface structures such as the setae from breaking, and they were frozen for at least 48 hours at -18 ˚C. A single larva was removed and defrosted for 5 minutes to reach room temperature and sprayed four times with a spray bottle delivering range of fine droplet sizes (preliminary measurements of water droplets indicted a range of 46-175 microns). There were two treatments, namely distilled water and/or a dilution of distilled water with 0.1% Triton X-100, which is a wetting agent. The nozzle of the spray bottle was positioned 50 cm away from a larva at a 90-degree angle. The larvae were examined with a NIS-Elements-D stereo microscope, 30 seconds after application, not exceeding a maximum investigation period of 30 seconds, and micrographs were captured at magnifications ranging from 4 to 40 times. No image processing was done. Micrographs were studied to observe differences in droplet entrapment between the control (distilled water) and mixture (distilled water with a wetting agent) applications.

## 3.4 Results

Droplets from both the water and wetting agent mixture were trapped by the surface structures of third- and fifth-instar larvae of all the species. Many water droplets were trapped on the cuticle surface, as well as by setae, of third- (Figures 3.5, 3.7, 3.9 and 3.11), and sixth-instar *S. frugiperda*, *H. armigera* larvae, *C. partellus* and *B. fusca* larvae (Figures 3.6, 3.8, 3.10 and 3.12). Droplets were trapped between surface structures and prevented from rolling off when they lost their mobility.

Although the wetting agent increased the superhydrophobic nature of the cuticle and caused droplets to roll off spontaneously from the cuticle surface, some droplets were still entrapped by the setae (Figures 3.5b, 3.6b, 3.8b, 3.9b, 3.10b and 3.11b).

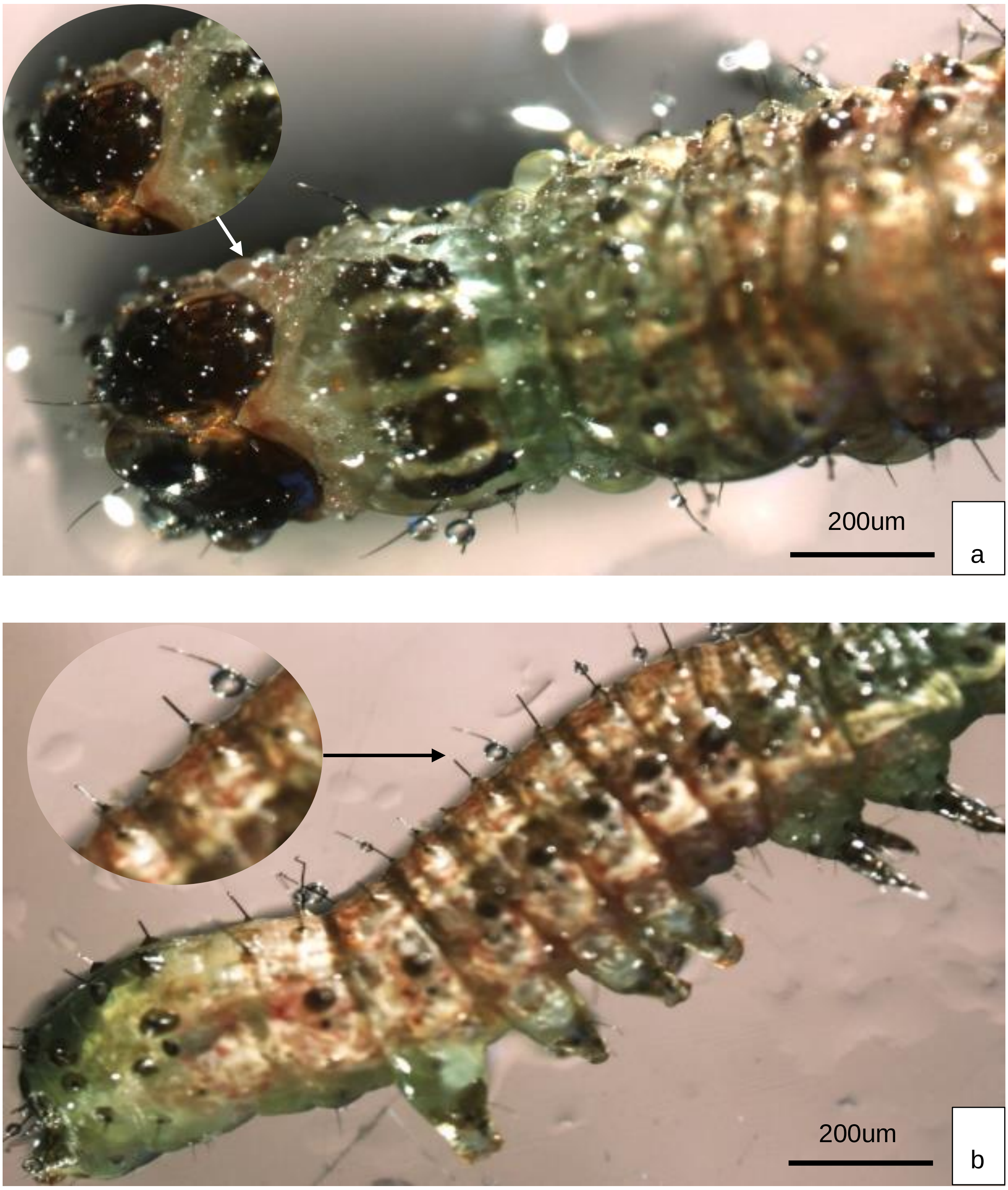

Figure 3.5 Micrographs showing droplet entrapment by third-instar *Spodoptera frugiperda* larvae. a) water, b) water and non-ionic wetting agent, Triton X-100.

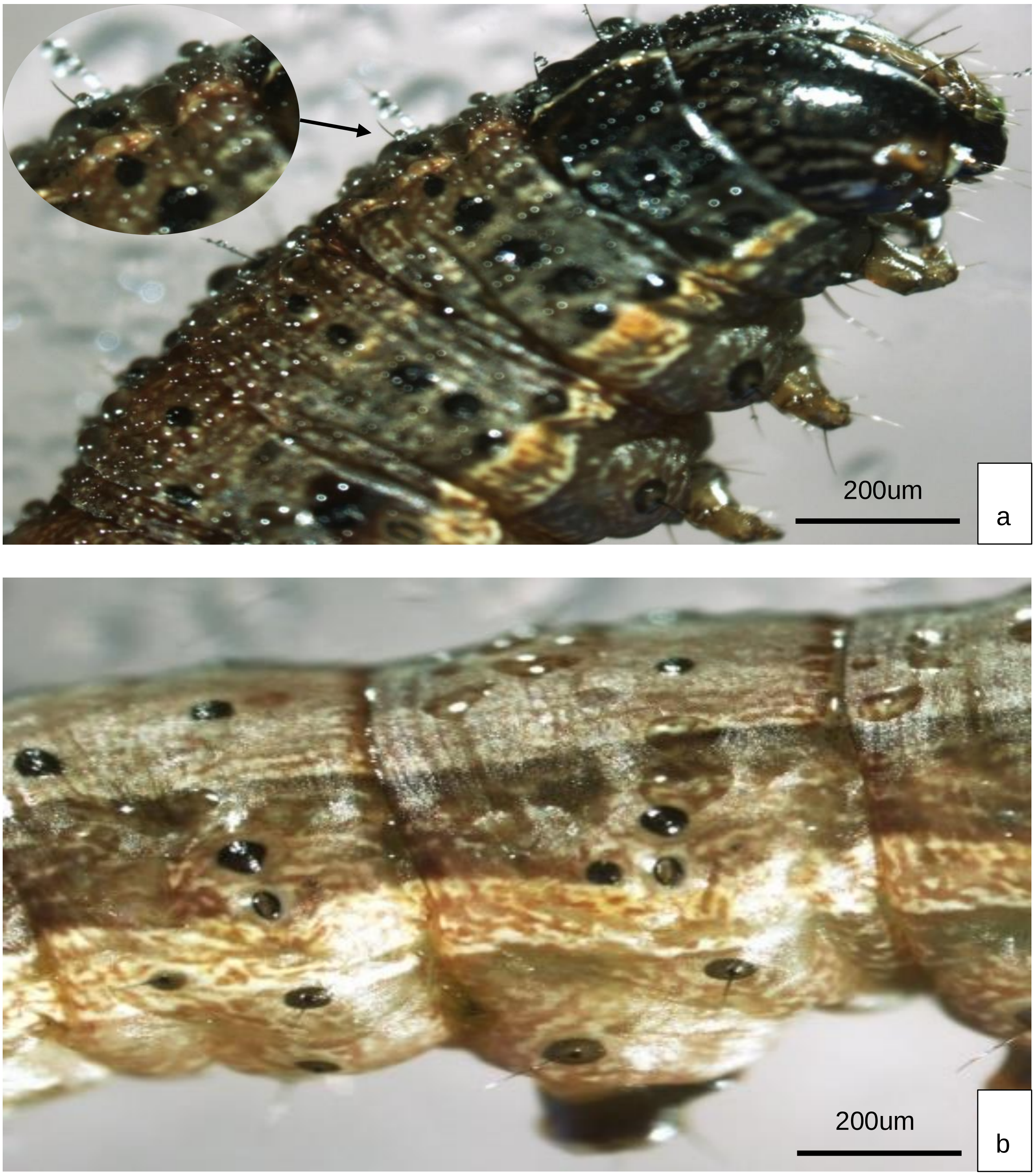

Figure 3.6 Micrographs of droplet entrapment by sixth-instar *Spodoptera frugiperda* larvae. a) water, b) water and non-ionic wetting agent, Triton X-100.

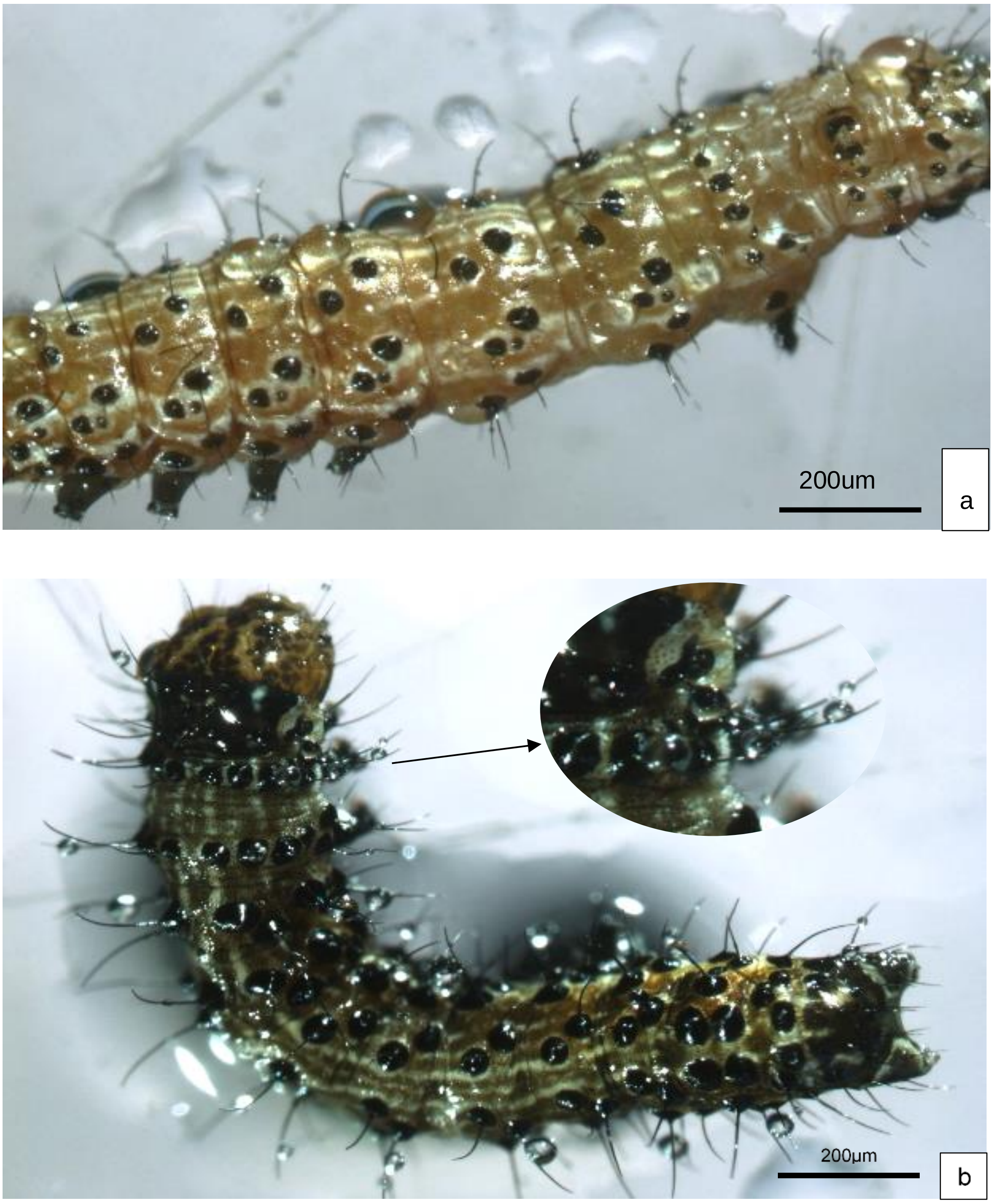

Figure 3.7 Micrographs of droplet entrapment by third-instar *Helicoverpa armigera* larvae. a) water, b) water and non-ionic wetting agent, Triton X-100.

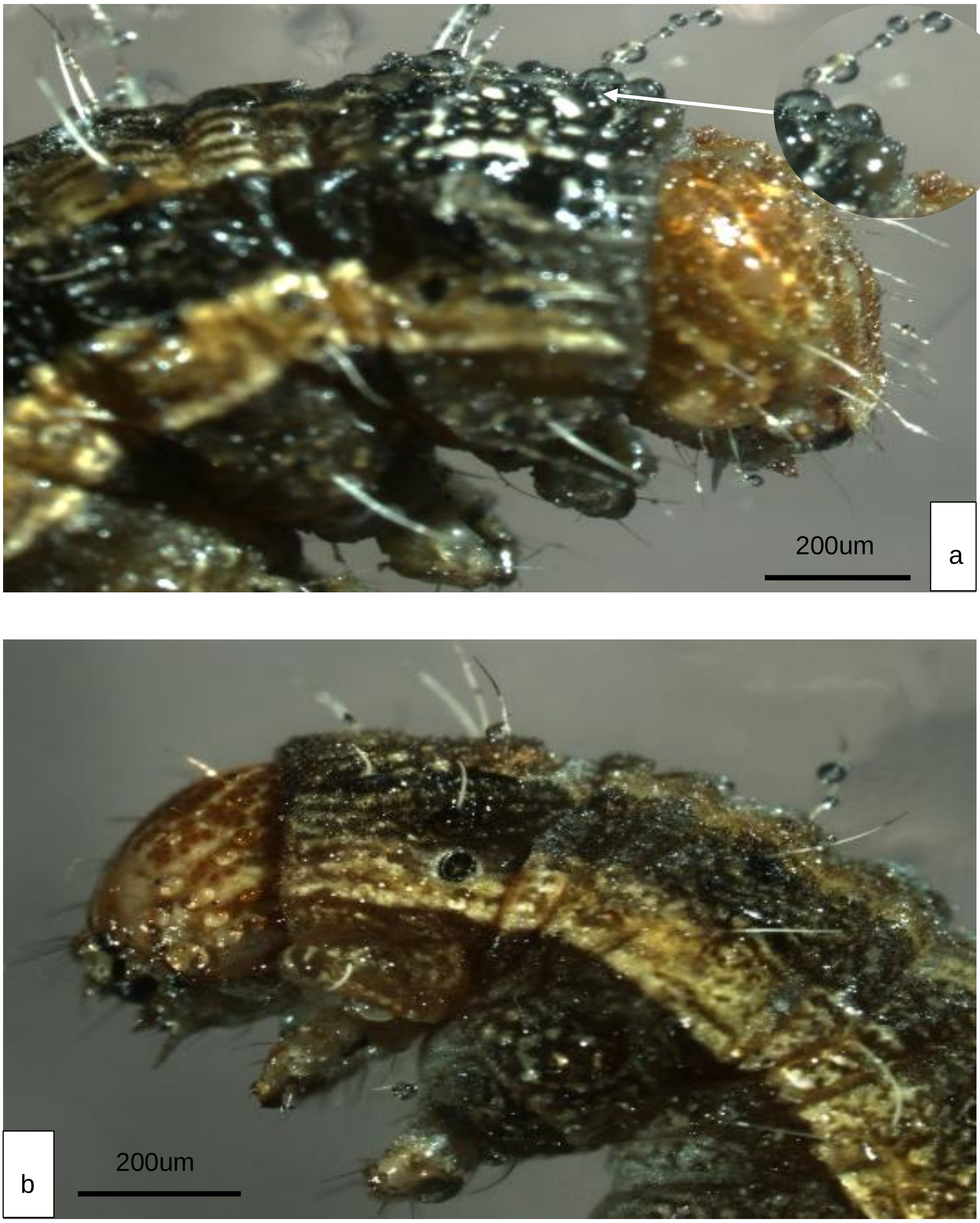

Figure 3.8 Micrographs of droplet entrapment by sixth-instar *Helicoverpa armigera* larvae. a) water, b) water and non-ionic wetting agent, Triton X-100.

The hydrophobicity of the cuticle to water droplets is evident from the droplet on the cuticle of *C. partellus* (Figure 3.9a), while application of the water-non-ionic wetting mixture provided a hydrophilic covering of the cuticle (Fig 3.9b).

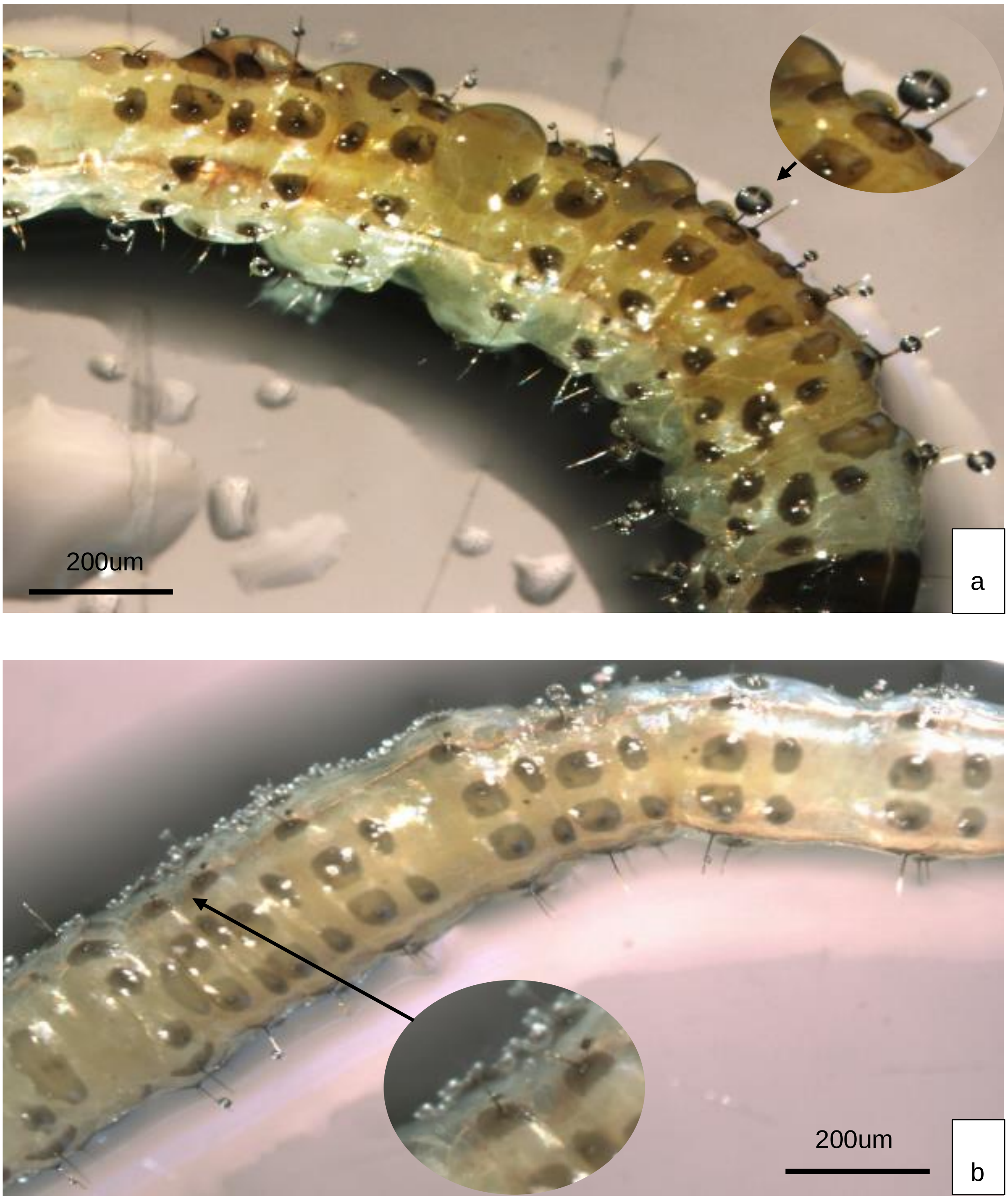

Figure 3.9 Micrographs of droplet entrapment by third-instar *Chilo partellus* larvae. a) water, b) water and non-ionic wetting agent, Triton X-100.

Figure 3.10 Micrographs of droplet entrapment by sixth-instar *Chilo partellus* larvae. a) water, b) water and non-ionic wetting agent, Triton X-100.

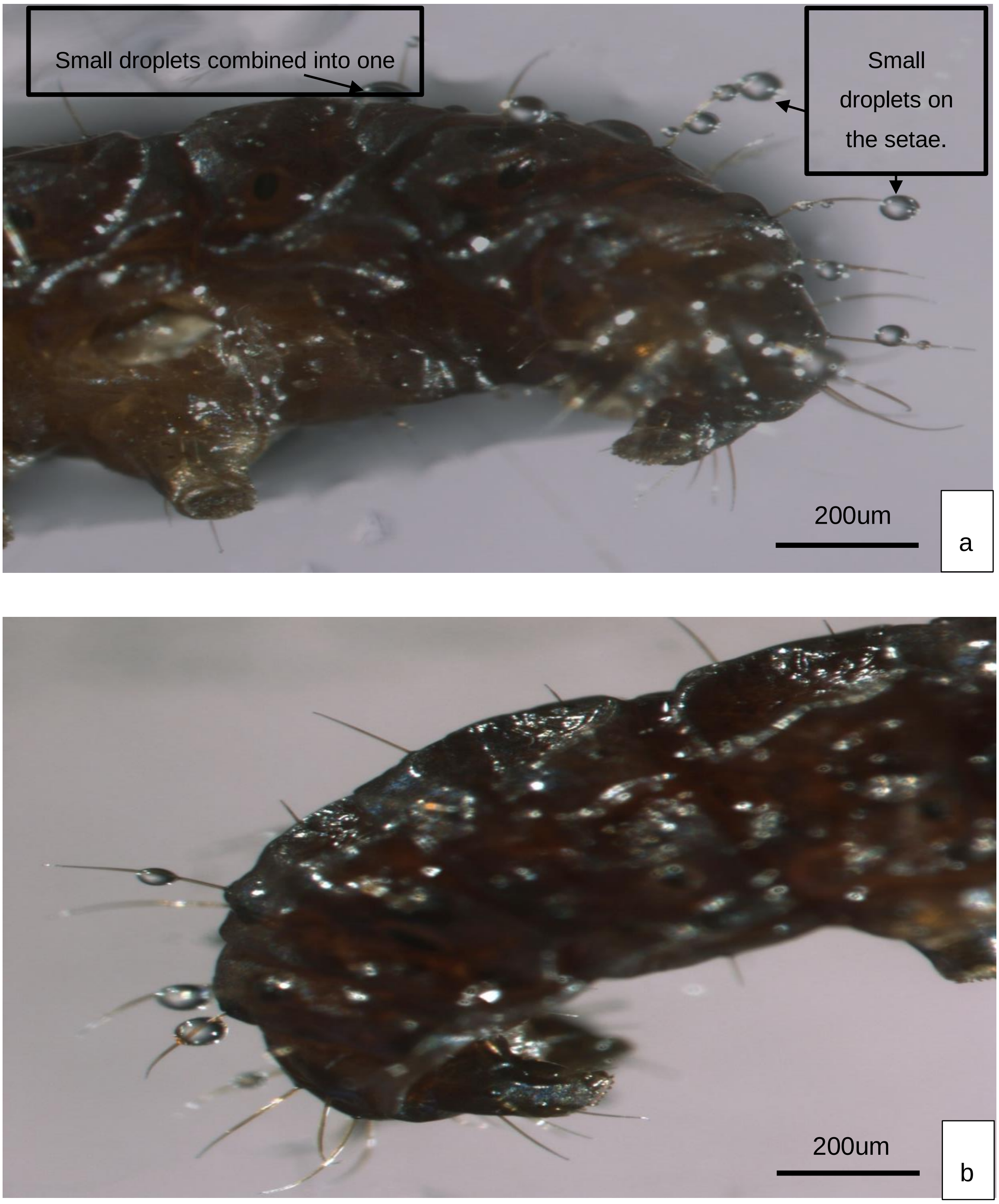

Figure 3.11 Micrographs of droplet entrapment by third-instar *Busseola fusca* larvae. a) water, b) water and non-ionic wetting agent, Triton X-100.

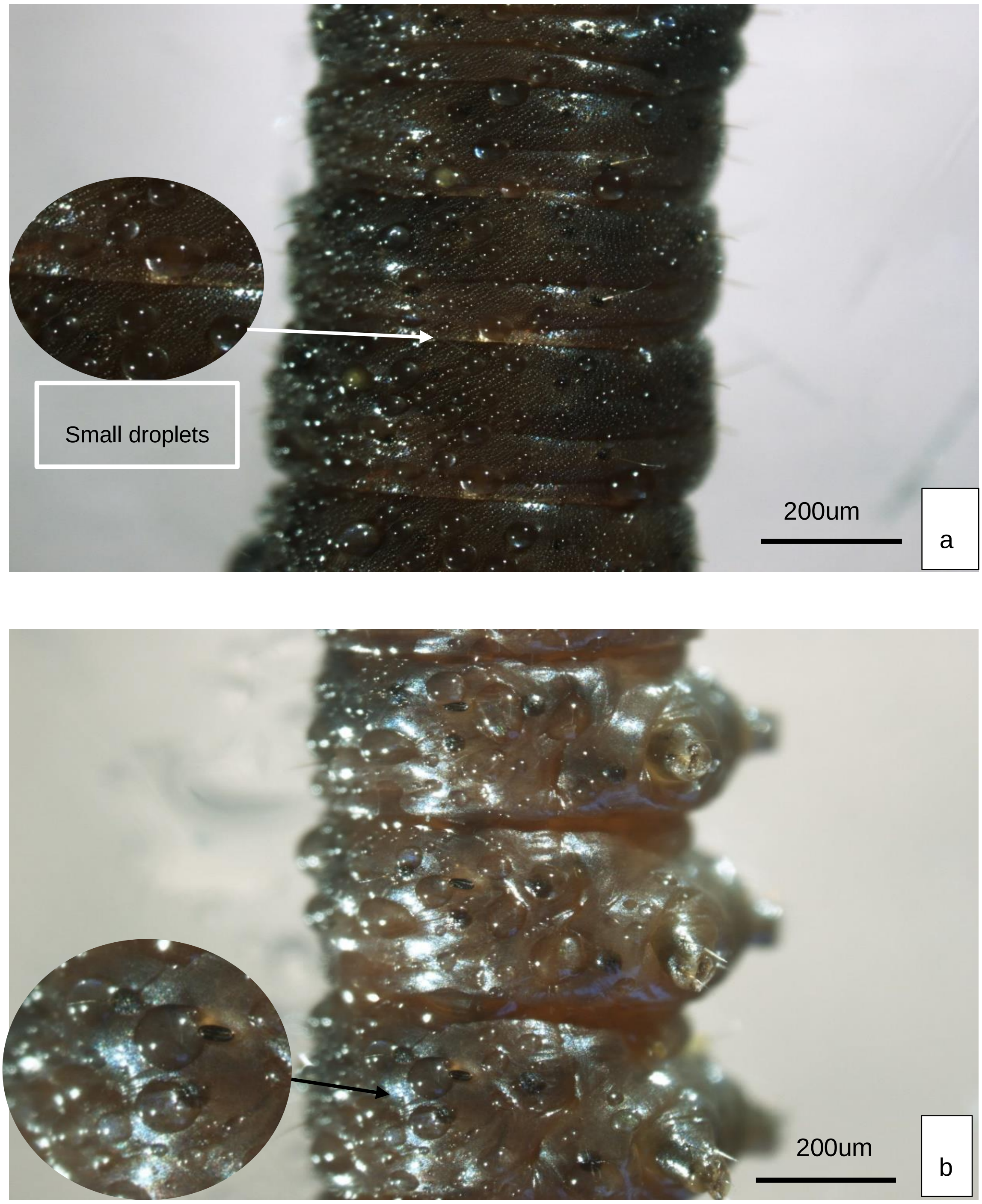

Figure 3.12 Micrographs of droplet entrapment by sixth-instar *Busseola fusca* larvae. a) water, b) water and non-ionic wetting agent, Triton X-100.

The entrapment of large droplets by setae, reduces the contact of the cuticle by the liquid (Figures 3.5-3.12). Smaller droplets also stuck to the sides of setae (Figures 3.11 a, b), preventing them from reaching the cuticle. Small droplets were present in direct contact with cuticle surface after spraying, as well as small droplets that combined into larger droplets (Figures 3.12a and 13a, b). Droplets were also present in the intersegmental membranes (Figures 3.12a, b and 13a).

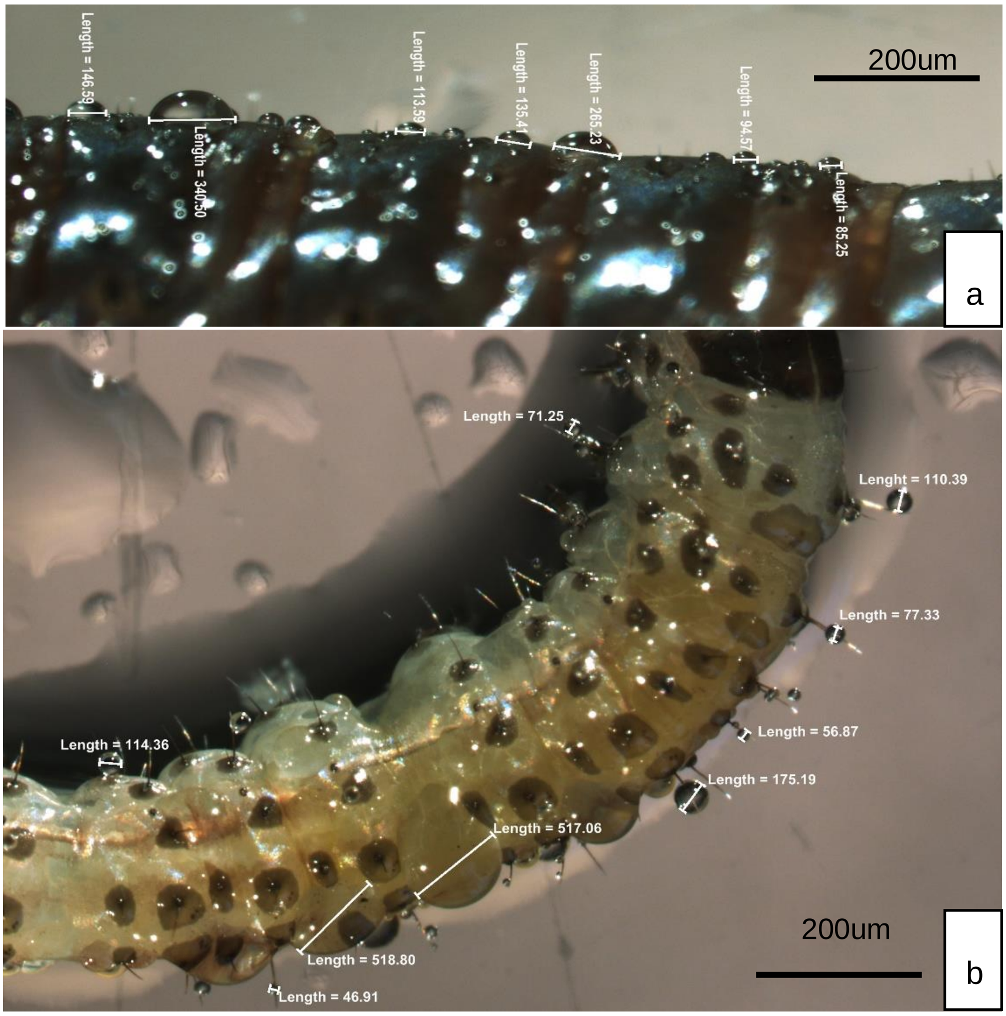

Figure 3.13 Micrographs of droplets of various sizes entrapped on the cuticle surface of a) *Busseola fusca* larvae and b) *Chilo partellus* larvae.

### 3.5 Discussion

Results from these observations confirmed that the cuticles of the third and sixth-instar larvae of *S. frugiperda, H. armigera, C. partellus* and *B. fusca* are hydrophobic and that many setae effectively trap water droplets. This may have important implications if control of these four lepidopteran pests is done by means of insecticide sprays. Droplets from the spray mixture should make contact with the cuticles of larvae as a mode of entry into the larvae to reach the target site.

Entrapment of droplets on the cuticles of the lepidopteran larvae in this study can also be explained by the classification of droplet types provided by Hu (2014). Water droplets on the cuticle surface of insects can generally be divided into two types. Firstly, droplets with low mobility (mist and other smaller droplets) that have a relatively high solid-liquid contact with the surface (type 1), and secondly, droplets with high mobility (much larger droplets) that are supported and held up by setae, making little or no contact with the underlying cuticle surface (type 2) (Figure 3.14) (Hu, 2014). Both these types of droplets can, however, come into contact with the cuticle surface, also observed in this study.

Figure 3.14 A schematic diagram presenting a type 1 droplet transition to a type 2 droplet being squeezed by the setae in contact as it grows larger (From Hu, 2014).

The transition from type 1 to type 2 droplets, by fusion between multiple single droplets, requires less energy compared to a droplet rolling through setae and by rough surfaces. Type 1 droplets can be squeezed by the pressure formed by setae with which they have contact (Fig. 3.14), when they enlarge and remain between the setae. This is a result of coalescing by type 1 droplets to form type 2 droplets, also observed on the cuticles of the lepidopterans observed in this study. It is more likely that contaminants will be picked up by transitioned type 1 droplets than by type 2 droplets, due to their availability, before they roll off the setae (Hu, 2014). However, although the cuticle is equipped with many structures reducing the contact area, it still comes into contact with liquid droplets.

Droplets entrapped on setae may cause direct wetting along the length of the hair to the bases, causing the droplets to grow bigger. As the size of a droplet increases, the centre of the droplet moves away from the surface of the setae (Figure 3.15) (Hu, 2014). The type 1 droplets then eventually transform into type 2 droplets, which allows these larger droplets to move towards the tips of setae, after which they detach from the surface and roll off.

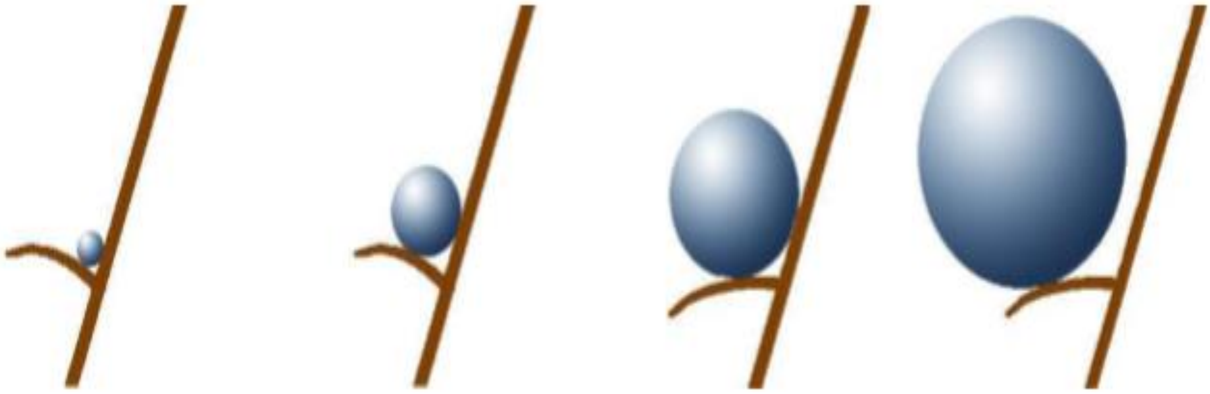

Figure 3.15 A schematic diagram presenting a type 1 droplet adhered to the base of a seta, and its transformation into a type 2 droplet (growing in size through coalescence) which detaches from the cuticle surface (From Hu, 2014).

The long setae are flexible, while shorter and stiffer setae are more densely packed (see for example chapter 2; Figure 2.9b). The long setae on the cuticles of lepidopteran larvae are the first line of defence against unwanted droplet interaction by creating adhesiveness and anti-wetting (Hu, 2014). The support of larger droplets (type 2) by the setae reduces the contact of liquids with the underlying cuticle surface. Smaller droplets stick to the sides of setae, also preventing them from making contact with the cuticle surface and assisting in the non-wetting of the membrane. Setae that are further apart allow droplets to have more contact with the underlying membrane (Hu, 2014). The underlying membrane region is more vulnerable to type 1 droplets that could potentially transform to type 2 droplets when combined (Fig. 3.14). The area around the bases of setae is, however, more accessible to type 1 (mist and smaller droplets) than to type 2 droplets (Hu, 2014).

The pressure load from larger droplets may cause the longer setae to collapse towards the shorter setae. Balance is, however, created by the loading force through the combination of stiffness from shorter setae and cuticle surface structures. Shorter setae contribute to anti-wetting by preventing smaller droplets from coming into contact with the underlying membrane (Perez-Goodwyn, 2009). Droplets become more mobile when the droplets combine and their mass increases. Smaller droplets may also coalesce with larger droplets, causing them to roll off (Watson *et al*., 2010a, b). The membrane area interacting with the droplet or combined droplets are therefore reduced by protuberances, when compared to a surface with little to no roughness (Watson *et al.*, 2011). Grooves between segments allow air pockets underneath droplets, which enable droplets to roll off more easily along the direction of the groove (Hu, 2014). The waxy surface covering the integument of insects (Hu, 2014), combined with the roughness of the cuticle, minimize the solid-liquid contact area by promoting air pockets in the topography that favour hydrophobicity (Holloway, 1970; Hu, 2014). This was also observed in this study for *S. frugiperda* (dome-shaped cones), *H. armigera, C. partellus* and *B. fusca* (cones). All these anti-wetting properties of an insect cuticle will therefore provide protection to the insect against insecticides that act through direct contact.

In this study, droplets applied from a distance above the cuticle surface membrane imposed kinetic energy, which caused setae to bend towards the membrane. As a result, a large area of the membrane was wetted before forces in the setae were restored that caused the droplets to be pushed away. Flexibility of setae and the impact from droplets dispensed from a distance above, caused the droplets to bounce off without dispersing, which is also promotes anti-wetting. However, if a variety of droplet sizes (extremely fine, fine and medium-sized droplets) reach the target area, the probability of droplets bouncing and/or rolling off the target is reduced. A variety of droplet sizes that reach the intended target area will increase the spreading of the insecticide over the insect's cuticle surface and increase wettability.

The efficacy of pesticide applications is affected by droplet sizes and the structure of individual droplets (Nuyttens *et al.*, 2007), which was also demonstrated in this study. Pesticide labels specify droplet sizes for effective application, which should therefore be adhered to. The type, orifice size and fan angle of nozzles are important factors in the application of pesticides that affect the efficiency of the pesticide application process (Nuyttens *et al.*, 2007). The Volume Median Diameter (VMD) refers to the midpoint droplet size (median), where half of the volume of spray is in droplets smaller, and half of the volume is in droplets larger than the median (Hofman and Solseng, 2004). Spray quality can range from extremely fine to ultra-coarse droplets, according to their VMD range.

Spray droplet sizes outside the recommended range might either drift away from the target area, or be too large and run and/or bounce off the target's surface (Boina *et al.*, 2013). The insecticide, target pest and method of application influences the optimum spray droplet size (Hanna *et al.*, 2009; Boina *et al.*, 2013). In this study, when no wetting agent was added to water, better coverage of the larval cuticles with smaller droplets was observed which is similar to results reported by  Wolf and Bretthauer (2009), who showed that smaller droplets, classified as fine or medium-sized, provide better coverage and increase the possibility of contact. For insecticide applications, droplets smaller than 250 micron in size are preferred (Grisso *et al.*, 2019), but the desired droplet size for insecticide applications is generally between 119-216 microns (Wolf and Bretthauer, 2009).

Pesticide efficacy on target surfaces will be higher if applied at the correct droplet range, and it is therefore an important factor to consider for insecticide applications (Hofman and Solseng, 2004). Application of droplets with unwanted sizes may reduce the spray quality (Nuyttens *et al.*, 2007), and can therefore affect the application outcome and cause control failure. Wettability of an insect cuticle can, however, be enhanced by a non-ionic wetting agent (Xu *et al.*, 2010).

Results from this study also confirmed that the non-ionic wetting agent caused an increase in the wettability of the cuticles of the respective species regardless the nature of the cuticle surfaces and structures. Droplet entrapment on the cuticles and cuticle structures such as setae of all four lepidopteran species, was reduced, and the wettability of the cuticle surface was significantly increased. A non-ionic wetting agent lowers the surface tension, which allows the droplets to spread over the contact area (Xu *et al.*, 2010).

Droplets within the recommended size range for insecticide application to which a non-ionic wetting agent is added, changes the Cassie-Baxter state of the cuticle to the Wenzel state with respect to the cuticle roughness (Byun *et al.,* 2009). This allows for complete wetting of insect cuticles with insecticides for effective control of pests.

## 3.6 Conclusion

Results from this study confirmed the hydrophobicity of cuticles of the third- and sixth-instar larvae of *S. frugiperda, H. armigera, C. partellus* and *B. fusca,* and that surface structures and cuticle topography affect droplet entrapment. Longer and closely spaced setae promote anti-wetting of the cuticle surface underneath through repelling water droplets, preventing the spreading of droplets over the cuticle surface. Shorter and more densely distributed protuberances (rough cuticle) reduce the contact area with the membrane by promoting the formation of air pockets in the topography that favour hydrophobicity. Wettability is enhanced by a wetting agent, regardless of the cuticle surfaces and structures. Adherence to insecticide label recommendations is therefore important to optimize effective control of insects. One of these important recommendations is the use of appropriate nozzles to ensure the desired droplet size for optimum wetting, as well as adding of an adjuvant, e.g., a wetter to improve spreading of the insecticide on the cuticles of insects.

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## CHAPTER 4

## CONCLUSIONS AND RECOMMENDATIONS

### 4.1 Conclusions

The most important lepidopteran pest species associated with maize in Africa are the stem borers, *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) (Mwalusepo *et al.*, 2015), as well as the Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), which invaded Africa in 2016 (Goergen *et al.*, 2016). The African bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a polyphagous pest, feeding on a variety of crops including maize, and can be found in high numbers if not controlled effectively (Wondafrash *et al.*, 2012). *Chilo partellus, H. armigera* and *S. frugiperda* are currently controlled in South Africa through spray applications of insecticides, while *B. fusca* are largely controlled by means of Bt maize. Labels of most of the insecticides (e.g. indoxacarb, chlorantraniliprole) currently registered for control of *S. frugiperda* in South Africa, indicate that application should not be done for control of larvae that are bigger than the third-instar (L3) (Labels available from: https://www.agri-intel.com/label-information/search-registration-information/).

An important entry route of insecticides into an insect's body is by means of penetration through the cuticle that provides a large target surface (Yu, 2008). Penetration resistance can, however, evolve through cuticle thickening or by altering of the cuticle composition (Tak and Isman, 2015; Balabanidou *et al.*, 2018). For example, reduced cuticular penetration has been reported in resistant strains of *H. armigera* from Australia (Gunning *et al*., 1991; 1995) and Thailand (Ahmad *et al*., 1999). Reduced efficacy of insecticides applied to later instar *S. frugiperda* larvae was reported by Lucchini (1977) cited in Fernandes *et al*. (2019). To investigate possible reasons for tolerance of later instar larvae to insecticides, cuticle thickness of third to sixth-instar larvae was investigated (Chapter 2). A significant difference in cuticle thickness between younger (third- and fourth-instar) and later instar (fifth- and sixth-instar) *S. frugiperda* larvae was demonstrated. A thickened cuticle layer can reduce the amount of chemicals that enters the body and delay insecticide penetration. The thickened cuticle may therefore contribute, together with other mechanisms, to older larvae being more tolerant to insecticides. Adherence to the recommendation provided on insecticide labels regarding the age of *S. frugiperda* larvae is therefore of utmost importance for effective control of this pest. Cuticular surface topography and protuberances, as well as the wax layer on the cuticles of all four lepidopteran species, affected the wetting ability of the cuticles of these larvae.

Scanning electron micrographs showed the pronotum cuticular surface topography to differ between the respective species (Chapter 3). Complex geometric (multiple protuberances that varied in shape) and simple micro- and nanostructures (dome-like and/or conical-shape), as well as hairs/setae (different in lengths), were observed. Protuberances on the cuticle of *H. armigera* include large, closely-spaced conical protuberances. The bumps present on the cuticular surface of *S. frugiperda* are absent on the cuticles of *H. armigera*, *C. partellus* and *B. fusca*.

For effective control of insect pests with contact insecticides, maximum contact with the insect's body must be achieved (Potts and Vanderplank, 1945). Protuberances perform multiple functions, important to insect survival. Surface roughness of micro- and/or nanostructures enhance non-wetting behaviour of insects (Watson *et al.*, 2017). Surface hydrophobicity should be reduced to overcome this protection mechanism for effective control with insecticides. Efficient application on target areas requires specific droplet sizes provided by different types of nozzles. It is therefore important to adhere to insecticide label recommendations with regard to droplet sizes to ensure effective coverage of the cuticle of a target insect. This can further be improved by adding a wetting agent to the spray mixture to reduce the surface hydrophobicity as demonstrated in chapter 3.

## 4.2 Recommendations

Results from this study provided basic information on the cuticle thickness of *S. frugiperda* and cuticle surface structure, as well as the potential role of external cuticle structures in droplet entrapment by four important maize pests, *viz*. *S. frugiperda*, *H. armigera*, *C. partellus* and *B. fusca*. This work can be repeated to consider not only the larval instar, but also the number of days spent in a specific instar, since the time after moulting may affect cuticle thickness. Testing of different wetting agents, with and without pesticides can also be done. Other mechanisms of resistance, for example detoxification by the different instar larvae should also be studied. It may, in conjunction with cuticle thickness, provide a better understanding of the lower susceptibility/higher tolerance by later instar lepidopteran larvae to insecticides. Field trials can be conducted to evaluate the efficacy of different insecticides with and without a surfactant added to the spray mixture.

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## Appendix A

## Declaration of language editing

### Language editing statement

To whom this may concern,

I, Prof. J van den Berg, hereby declare that the thesis titled: “Potential effect of cuticle thickness and external structures of Lepidoptera larvae on mode of entry of insecticides” by M. Benade has been checked for language correctness and spelling, as far as possible, by the supervisors. The literature references are considered the responsibility of the student.

J van den Berg

Prof. J van den Berg

3 December 2021