CHAPTER 3.2.4.

INFESTATION OF HONEY BEES WITH *AETHINA TUMIDA* (SMALL HIVE BEETLE)

SUMMARY

***Description and importance of the disease**: The small hive beetle,* Aethina tumida (*Murray 1867*) (Coleoptera: Nitidulidae), *is a parasite and predator of honey bees. Adults and larvae of small hive beetles feed on honey bee brood, honey and pollen. While feeding on food stores the remaining honey is fermenting and the comb is destroyed. The beetles can promote structural collapse of the nest and cause the adult honey bees to abscond from severely infested colonies. The extent of beetle-associated damage depends on climate, colony strength and other conditions. Small hive beetles tend to be more problematic in areas with warm temperatures and high humidity. The small hive beetle can be a serious problem in honey-extracting facilities where stored comb, honey and wax cappings are potential feeding and breeding areas. Beetle development from egg to adult requires 3–12 weeks, depending on humidity, temperature and food availability. The flying adult beetles actively infest honey bee colonies of all strengths and sizes.*

***Identification of the agent:** An infestation by the small hive beetle can be recognised either indirectly via colony-wide damage associated with the beetle or directly via eggs, larvae and adults. An early diagnosis can be made after opening the colony and finding adult beetles under the colony lid, on the bottom board, or hiding in the combs (especially peripheral combs). Definitive diagnosis at the laboratory is based on morphological examination under a stereomicroscope. Confirmatory testing can be done by real-time polymerase chain reaction.*

***Serological tests:** Serological tests are not applicable.*

***Requirements for vaccines:** No vaccines are available.*

A. INTRODUCTION

The small hive beetle (hereafter referred to as "beetle"), *Aethina tumida*, order *Coleoptera,* family *Nitidulidae* (Murray, 1867), is native to sub-Saharan Africa (Hepburn & Radloff, 1998) but has been found in various regions of the world over the past few decades. *Aethina tumida* was first detected in the United States of America in 1996. Since then it has spread to Canada and a number of countries in South and Central America. *Aethina tumida* has also been found in Australia, Egypt, Italy, Korea and the Philippines (WOAH WAHIS Interface, database accessed on 20/06/2017; Lee *et al.,* 2017).

1. Life cycle

The infesting small hive beetle adults mate in the colony and the female beetles oviposit several eggs in typical clutches in small cracks or within capped brood cells (Cuthbertson *et al.,* 2013; Ellis, 2005; Lundie, 1940). In some situations, more than 1000 adult beetles may occur within a colony (Elzen *et al.,* 1999). Adult beetles can survive up to 12 months (records indicate up to 16 month in laboratories; Somerville, 2003), but females die quickly when ovipositing on a daily basis (Neumann *et al.,* 2016). Females can oviposit about 1000 eggs in their lifetime (Lundie, 1940), though Hood (2004) suggested the upper limit may be 2000 eggs. Successful egg emergence is correlated with relative humidity, with fewer eggs hatching at a relative humidity of <50%. The larvae emerge from the eggs after 1–6 days (most within 3 days) and feed on pollen, honey and bee brood (Lundie, 1940; Schmolke, 1974). Adult beetles can be fed by worker bees via trophallaxis, especially while confined in bee-guarded "prisons" (Ellis, 2005). Larval development usually takes about 2 weeks (8–29 days depending on food availability and temperature; de Guzman & Frake, 2007; Ellis *et al.,* 2002b; Lundie, 1940; Schmolke, 1974). Following this, the larvae reach the

wandering phase and leave the colony to pupate in the soil surrounding the colony (Lundie, 1940). Pupation takes about 2–12 weeks depending on temperature and soil moisture (Ellis et al., 2004). Emerging adults leave the soil and can fly to search for new host colonies, thereby completing their life cycle. In laboratory conditions, the small hive beetle can survive and reproduce on ripe or rotten fruits (Buchholz *et al.*, 2008).

2. Impact of the pest

Small hive beetle is seldom a serious problem in Sub-Saharan Africa. The reasons for the apparent difference in its impact on colonies within its native range and those in its new ranges are not well understood (Ellis & Hepburn, 2006). They may include quantitative behavioural differences between African and European honey bee subspecies, different beekeeping techniques, climatic differences, or escape from natural enemies, among other plausible hypotheses (Hood, 2004; Neumann & Elzen, 2004).

While bee colony damage due to adult beetles is relatively minor, the adults can cause colonies to abscond (i.e. the adult bees completely abandon the nest; Ellis *et al.,* 2003). If not prevented, larval feeding behaviour is often associated with fermentation of stored honey, causes severe damage to combs and often results in the full structural collapse of the nest (Lundie, 1940). Economic losses also can be associated with beetle infestations in the honey-extracting facility. Environmental conditions generally associated with extracting facilities, such as high temperatures and humidity, provide optimal conditions for beetle development. Cryptic low-level reproduction may also occur either in the debris or underneath hive inserts without any signs of colony damage (Spiewok & Neumann, 2006).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of infestation with Aethina tumida *and their purpose*

Method	Purpose					
	Population freedom from infestation	Individual animal or bee nest freedom from infestation prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infestation – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent						
Morphology	+++	+++	+++	+++ (adults) + (larvae)	+++	–
Real-time PCR	++	++	++	++	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction,

1. Field detection

1.1. Adult beetles

The first sign of an infestation by the small hive beetle is the occurrence of adult beetles (Figure 8). Adult beetles are ~5 mm long and ~3 mm wide, with females being slightly longer than males (Ellis *et al.,* 2001). The adults are dark brown to black (lighter shortly after eclosion). During inspections, adults avoid sunlight, hide, and can be observed while running for cover into corners or similarly over the combs. Adults can be confused with other nitidulid beetles, which can also be associated with colonies (see Section 2.2.3 below for details; also Ellis *et al.,* 2008, Marini *et al.,* 2013 and Neumann & Ritter, 2004).

1.2. Beetle eggs, larvae and pupae

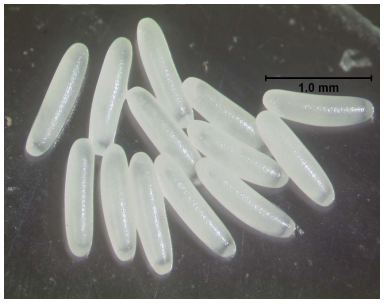


Fig. 1. Small hive beetle eggs. Photograph by Josephine Ratikan, University of Florida.

Small hive beetle eggs (Figure 1) are white, ~1.4 × 0.26 mm (length × width); ~2/3 of the size of a honey bee egg, and are deposited in clutches in cracks, on the bottom board, on the combs and underneath the cappings of sealed brood cells. Larvae (Figure 2) are whitish, up to ~1 cm long (wandering phase), have three pairs of legs, and have dorsal spikes. Larvae can be found mining in the wax combs (Lundie, 1940) or in colony debris (Spiewok & Neumann, 2006). Larval infestations are typically associated with a rotten smell due to death of honey bee brood or fermentation of the stored honey. Wandering larvae often leave smear trails (or "slime") inside and outside the colony (Figure 3). Once in the ground, the larvae excavate small pupation chambers (Figure 4) 1–20 cm deep in the soil (Pettis & Shimanuki, 2000), develop into pupae (Figure 5, whitish to dark brown depending on age, ~5 mm long and 3 mm wide) and then into adults. Most larvae tunnel into soil that is <180 cm from the colony (Pettis & Shimanuki, 2000).

Fig. 2. Dorsal (left) and ventral (right) view of a small hive beetle larva. Photographs by Josephine Ratikan, University of Florida.

Fig. 3. Comb damage attributed to the feeding/crawling habits of small hive beetle larvae. Notice the "slime" on the frame (i.e. the wax comb looks "wet" and it "glistens"). This is caused by the fermentation of honey, which is moved around the comb by crawling larvae. Beetle larvae can be seen in cells in the centre of the comb, where brood was present originally. Photograph credit, University of Georgia.

Fig. 4. Small hive beetle larvae that has tunnelled into the soil and hollowed out a chamber in which to pupate. Photograph credit, University of Georgia.

Fig. 5. Small hive beetle pupa (ventral view). Photograph by Lyle Buss, University of Florida.

It is difficult to find beetle eggs in a colony, especially at low levels of infestation. Cracks or crevices around the nest should be investigated or capped brood cells that have small holes in the cappings, possibly indicating that a female beetle has punctured the capping and oviposited within the cell. Small

hive beetle pupae can be found by sifting the soil around the colony and looking for the pupal chambers or the pupae themselves.

1.3. Visual inspection of colonies

When monitoring honey bee colonies for the presence of small hive beetles, an examination of the hive may provide an early indication of infestation. In countries still free of *A. tumida*, it is recommended to monitor sentinel apiaries in zones at risk of an introduction, to detect an infestation at an early enough stage to eradicate it (Chauzat *et al.,* 2016). Furthermore, if the intent is to eradicate *A. tumida,* sentinel colonies should be placed in the location of infested apiaries that have been previously sanitised. Such sentinel colonies act as a bait to attract free-flying beetles and must be in place immediately after all colonies are destroyed, as adult beetles staying outside colonies might survive the eradication and spread further to other hosts nearby.

It is important to adapt the method of visual inspection to limit the expected spread of *A. tumida*, as colony manipulation might induce disorder and robbing (where bees steal honey from other colonies) or adult small hive beetles may occasionally leave the colonies during manipulation. The currently used visual inspection methods in infested areas are most feasible in the field and easy to carry out for anyone trained to manipulate a beehive (EFSA, 2015; Neumann *et al.,* 2016). Colony manipulation requires a certain minimum training and awareness of small hive beetle biology and morphology, to correctly inspect field-colonies and to quickly detect and recognise damage caused by the different life stages of *A. tumida*. A colony inspection begins right at the entrance of the hive and relies on the rapid but meticulous examination of the lid, the inner cover, the frames and the bottom board. The following recommendations are from the EFSA scientific opinion (EFSA, 2015).

1.3.1. Colony inspection method (EFSA, 2015)

i) Remove the lid and check for the presence of adult beetles running away.

ii) Remove the inner cover and check both sides. Check also the top of the frames for running adults.

iii) Remove the frames from the hive one by one. Each side of the frame should be quickly observed to check the presence of adult beetles, larvae, eggs and damage. The first frame can be left outside the body of the hive to make it easier to handle the other frames. Subsequent frames should be put back into the body or super (the part of the hive in which bees store honey) to prevent robbing in the apiary during the examination.

iv) Beetles can hide inside the cells of combs. It is also important to examine the lid, the bottom board, the side faces, corners, interstices of the hive and hive components.

If robbing is unlikely, the super can be examined by placing it on the inverted lid of the hive in a sunny spot. Adults will escape from the sunlight and retreat down into the lid. After about 10 minutes, the presence of adult beetles in the lid can be checked by lifting the super (Zawislak, 2014). If there is a risk of robbing, the super should be inspected in the same way as the body of the hive, i.e. comb by comb, by replacing each frame in the box after its examination. During the examination of the body, the super can be placed on a reversed lid, so that no bees or beetles can escape (Spiewok *et al.,* 2007).

To improve the sensitivity of the visual inspection, the hive can first be removed from its original position, then opened and replaced by an empty hive (Neumann & Hoffmann, 2008; Spiewok *et al.,* 2007). Each frame is then removed and examined for beetles for the first time. The bees are then shaken into an empty box and the comb is inspected for a second time for beetles, this time in the absence of bees, before being placed into the new hive. Once all the frames have been examined, the original hive box and bottom board are inspected. However, this method is more time-consuming, and requires additional beekeeping equipment and therefore is not suitable for routine monitoring of small hive beetle infestation in large apiaries. It is however, recommended for health certification to demonstrate the absence of *Aethina tumida* infestation in a colony.

A method, originally described in Canada, uses a white 12-litre bucket fitted with a wire-mesh screen (about 6 mm) fitted halfway down the depth of the bucket. The bottom of the bucket is covered with a thin layer of vegetable oil. The frames are shaken inside the bucket and bees are stopped unharmed by the wire-mesh whereas beetles fall into the vegetable oil. Field data suggest that this method is more sensitive than simple visual inspection when the infestation level is low[1].

1 http://www.omafra.gov.on.ca/english/food/inspection/bees/2011-shb-report.htm

Another method for colony examination is described below. The method can be used to search for beetle adults and larvae, if larval infestations are moderate to high (Ellis *et al.,* 2002a, Ellis & Delaplane, 2006), but should not be used if eradication is planned, as it might increase the number of free-flying beetles.

Fig. 6. Inspecting a colony for adult small hive beetles. The inspector on the right has shaken the bees onto a piece of plywood. Both faces of the framed comb were then bounced onto the wood to dislodge the beetles from the cells. The inspector on the left is shifting through the adult bees and using a mouth aspirator to collect the beetles. Photograph by Keith Delaplane, University of Georgia.

Notes:

i) This procedure is best accomplished with two people, one to work the colony and the second to collect the beetles if quantification is desired. Only one person is needed if beetle detection is the sole desired outcome.

ii) Some beetles inevitably fly away or hide from view during this procedure. The number of beetles that escapes is presumed to be low (<5%).

iii) This procedure is best used for qualification of adult beetles. However, larval beetles can also be found this way.

a) Place a sheet of opaque plastic (~2 × 2 m, preferably white or light in colour) or plywood in front of the colony which you want to inspect for beetles.

b) Lightly smoke the colony.

c) Remove the lid from the colony and bounce the lid on the plywood. This should be done to dislodge all adult bees and beetles adhering to the lid.

d) A second individual (the beetle collector) should comb through the bees (this can be done with the hand or using a small stick) and collect all adult beetles seen using an aspirator. All bees on the plywood should be inspected as beetles can easily be concealed by clusters of bees (Figure 6).

e) Remove the outermost frame in the uppermost super (i.e. the uppermost “box” containing bees) and shake the bees from the frame onto the plywood.

f) The beetle collector should repeat step d.

g) Once the bees have been shaken from the frame, the frame should be turned onto its face and bounced against the plywood to dislodge adult beetles from the comb. This step should be repeated two-to-three times for both sides of the frame.

h) The beetle collector should repeat step d.

i) The individual working the colony should repeat step g to all frames in the uppermost super and then bounce the empty super on the plywood. This step should be repeated for all supers, all frames, and the bottom board of the colony.

The latter two described colony inspection methods are time-consuming and there is a very high risk of inducing disorder and robbing in the apiary (EFSA, 2015) and a risk of making the beetles fly away.

1.4. Colony examination using traps

The use of traps for small hive beetle detection has been described in the Guidelines for the surveillance of the small hive beetle (*Aethina tumida*) infestation (updated version: April 2016) developed by the European Union reference laboratory for honey bee health (Chauzat *et al.,* 2016).

The principle of most small hive beetle traps is to offer shelter from bee aggression by providing a passage that is large enough for beetles but too small for bees to enter. In their attempt to get away from the bees that chase them, *A. tumida* adults will enter the trap in which oil or veterinary medicines may be used as a killing agent. Sometimes this principle is combined with the use of bait that can increase trap

efficacy. The position of the trap inside the hive is important and has to be adjusted to the hive type and to climatic conditions as beetles may hide on the bottom boards or in the periphery of the colony if climatic conditions are warm but tend to stay within the clustering bees when temperatures are low. Therefore, traps are available for all positions in the hive and all of them should be checked regularly during apiary visits.

Traps that are placed between frame top-bars consist of small containers that are covered by a grid. These kinds of traps are usually filled with vegetable oil (diatomaceous earth was successfully tested in the laboratory; Cribb *et al.,* 2013) and they were shown to be effective in North American conditions (Bernier *et al.,* 2015). The trap is placed between the top-bars of two frames, close to the brood nest or the winter cluster. When visiting the colony, traps are examined for the detection of any beetles. If the container is transparent, this observation is easy and straightforward. It was shown using these kinds of trap that bees might seal the openings with propolis thereby reducing their efficacy (Bernier *et al.,* 2015). Care must also be taken to prevent any oil spill.

In the warm season, traps placed on the bottom boards or modified bottom boards could be used for the detection of adult small hive beetles. Modified bottom boards usually consist of an oil filled tray that is placed underneath a grid or a mesh-screen. If the tray is covering the whole bottom, the hive must be levelled, but some of these traps cover only parts of the bottom board. Although these traps work well, they require hive-modification and therefore are mainly feasible for stationary beekeeping.

Many traps were invented for use on the bottom board. One example without any bait and killing agent is a 4 mm corrugated plastic strip (Figure 7). It was shown to be effective in field trials in Australia and the USA. The corrugated plastic suits the thigmotactic behaviour of *A. tumida* as it consists of square flutes, big enough for the beetle to get in, but too small for the bees to enter (approx. 4 × 4 mm). The hive does not need to be opened as traps are placed inside the hive through the entrance. It is important to properly place the trap in contact with the solid floor of the hive. If not, beetles can seek refuge in the space located between the trap and the floor. For optimum use, traps should be left in hives for a minimum of 48 hours before they are checked. The trap should preferably be made of a transparent material so that beetles can quickly be detected (Schaefer *et al.,* 2008). Other bottom board traps bring the beetle in contact with killing agents inside the traps. Chemicals can have high efficacy but there is always a risk that resistant strains may develop, residues might accumulate in honey or other hive products or that the chemicals could spread to the bees leading to adverse side effects on them. An alternative to the use of chemicals is diatomaceous earth or traps that just use adhesive film.

A biomechanical way of trapping small hive beetle inside the hive is the placement of kitchen wipes or similar material on top of the frames. The bees shred this material into fibres in which the beetles become entangled. This very simple to use and economical control method has the advantage of functioning without any lethal substance, but fibres might also end up in the honey.

Fig. 7. Corrugated plastic insert used to detect adult small hive beetles. The plastic insert contains square flutes (left) in which adult beetles hide when inserted onto the bottom board of a colony, through the colony entrance (right). The insert must be used in conjunction with a traditional solid bottom board rather than a screened bottom board. Photographs by James Ellis (left) and Stephanie Kimball (right), University of Florida.

In low infested areas it is especially recommended to always undertake a combination of visual inspections and traps to increase the sensitivity of detection. Depending on the seasonal conditions, it may be decided to use either visual observation or traps, but, whenever possible, combining them both is best. In apiaries where inspections are frequently undertaken (sentinel apiaries), surveillance traps may be used. For single inspections, visual inspections may be best because of the higher detection sensitivity and to avoid a return visit to check the trap.

For a more detailed description of different traps refer to EFSA, 2015 and Neumann *et al.,* 2016.

2. Laboratory identification

Rapid and reliable diagnosis is crucial for the implementation of sanitary measures and to avoid spread in non-infested territories. Suspect field specimens should be sent to official laboratories for confirmation of *A. tumida* identification. Morphological identification is fast and inexpensive, and does not require sophisticated equipment. Confirmatory testing can be done by molecular methods (polymerase chain reaction [PCR]), and is particularly useful for larval identification or when specimens are damaged.

2.1. Special precautions required for sample handling

The specimens to be identified are collected in or near honey bee hives (for example, in colonies, beekeeping equipment or queen cages).

Suspect specimens should be killed before submission to the laboratory e.g. in 70% ethanol. Denatured ethanol should not be used where molecular methods are to be used because of possible PCR inhibition. Alternatively, specimens can be stored overnight at –20°C to kill the specimens.

On arrival at the laboratory packages should be opened in containment conditions. If the specimens are found to be alive on arrival, the submission should be placed at –80°C for approximately 1 hour before any work can be done with them. This procedure immobilises the specimens, which can subsequently be stored in 70% ethanol.

2.2. Morphological identification of adults and larvae

The test method aims to identify *A. tumida* by examining the external appearance of adults or larvae specimens in the laboratory. It consists of the visual examination of specimens noting morphological characteristics specifically selected to differentiate *A. tumida* from other nitidulid beetles and wax moth larvae, commonly found in honey bee colonies, queen cages or beekeeping equipment.

2.2.1. Equipment and reagents

Classical entomological materials are required for the morphological identification of *A. tumida*, including a stereomicroscope (or a magnifier), entomological tweezers, evaporating dishes (glass, plastic or porcelain) or Petri dishes, capped tubes for specimen storage, 70% ethanol (not denatured ethanol).

2.2.2. Test procedure

A general observation of the specimens should be made by placing them in a dish and checking for homogeneity (using a magnifier or stereomicroscope as necessary). If they are of uniform type, the samples can be processed further. If they are not uniform (i.e. multiple species may be present) then samples should be taken of each type present for further identification. When possible, select undamaged samples for further analysis, using entomological tweezers.

Microscopic examination should be done at different magnifications to visualise the critical identification criteria (see Section 2.2.3 below). The size of the specimens should be measured. Samples can be compared with reference specimens if available. After examination, beetles are stored in 70% ethanol.

2.2.3. Guidelines for the identification of *Aethina tumida*

Differentiation should be made between *A. tumida and* other non-pest nitidulid beetles that can be found in honey bee hives, for example: *Cychramus luteus*, found in Europe, that mainly feeds on pollen (Neumann & Ritter, 2004), *Carpophilus lugubris*, found in hives in Italy (Marini *et al.,* 2013), and *Glischrochilus fasciatus, Lobiopa insularis, Carpophilus dimidiatus* and *Epuraea corticina* found in hives in the United States (Ellis *et al.,* 2008).

The larvae of *A. tumida* can also be mistaken for larvae of the lesser wax moth, *Achroia grisella,* or the honeycomb (greater wax) moth, *Galleria mellonella*. These lepidoptera are generally found in colonies and on beekeeping equipment.

2.2.3.1. Adult form

Identification of adult *A. tumida* is based on the following morphological criteria: (Figures 8 and 9)

1. Body divided in three parts: head, thorax and abdomen
2. Three pairs of legs
3. Presence of elytra
4. Dimensions: length: 5–7 mm; width: 3–4.5 mm (approximately)
5. Colour: reddish brown when newly hatched, turning dark brown to black in adulthood
   Presence of a lighter band around thorax and abdomen (optional criterion)
   Note: The colour may change with environmental conditions and conservation of the specimens
6. Club-shaped antennae
7. Sharp posterio-lateral angles of the pronotum
8. Elytra not covering the entire abdomen

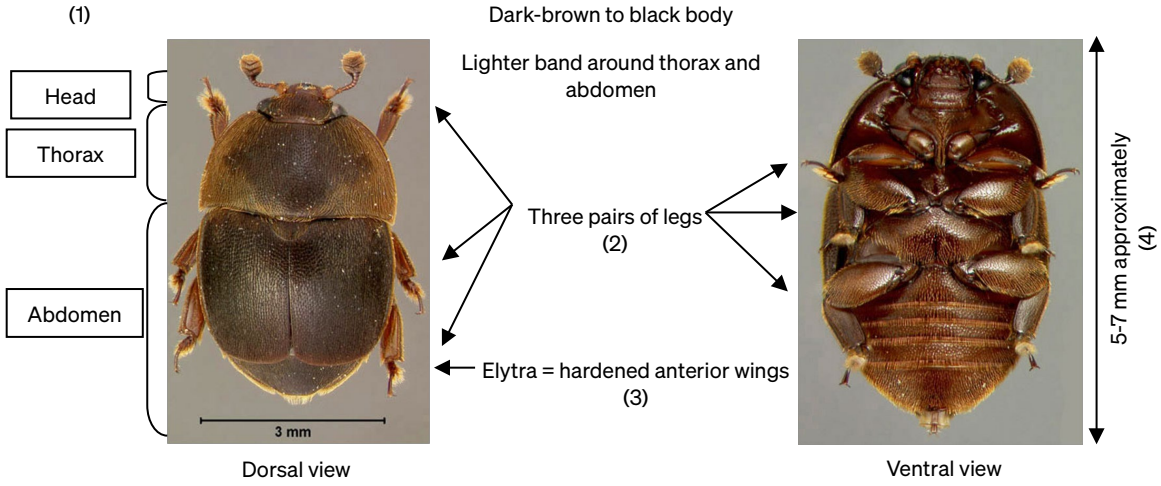


***Fig. 8. Small hive beetle,* Aethina tumida.**
Photographs by Lyle Buss (left) and Josephine Ratikan (right), University of Florida.

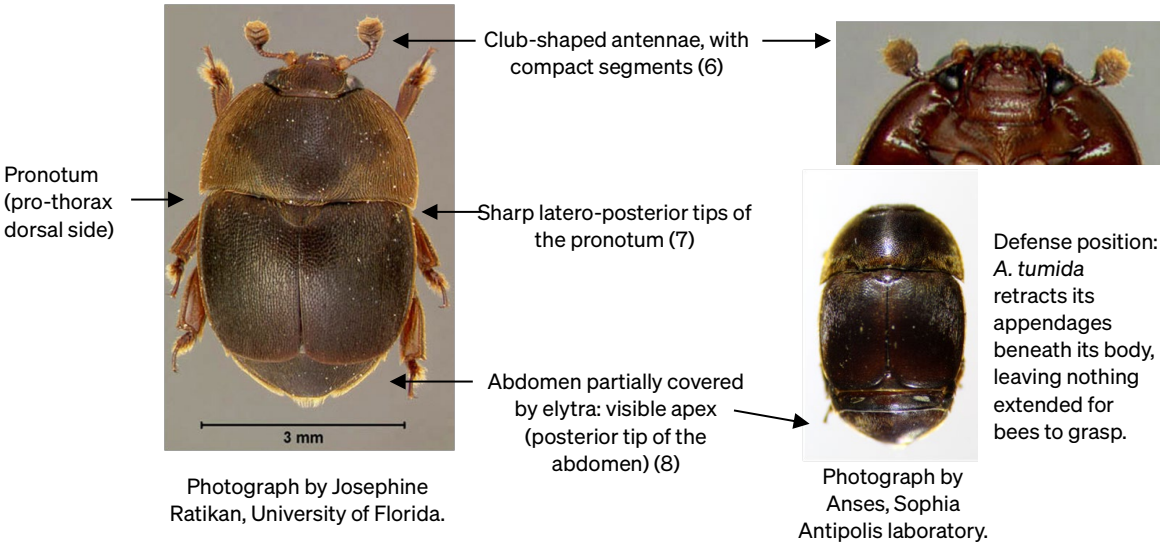


Fig. 9. Small hive beetle,* Aethina tumida *Murray.

For differential diagnosis, *Cychramus luteus* is shown below with the following features (Figure 10; Neumann & Ritter, 2004):

elytra completely covers the abdominal apex;
antennal clubs are looser with detached segments;
latero-posterior tips of the pronotum are not sharp;
colour of the body is light-brown.

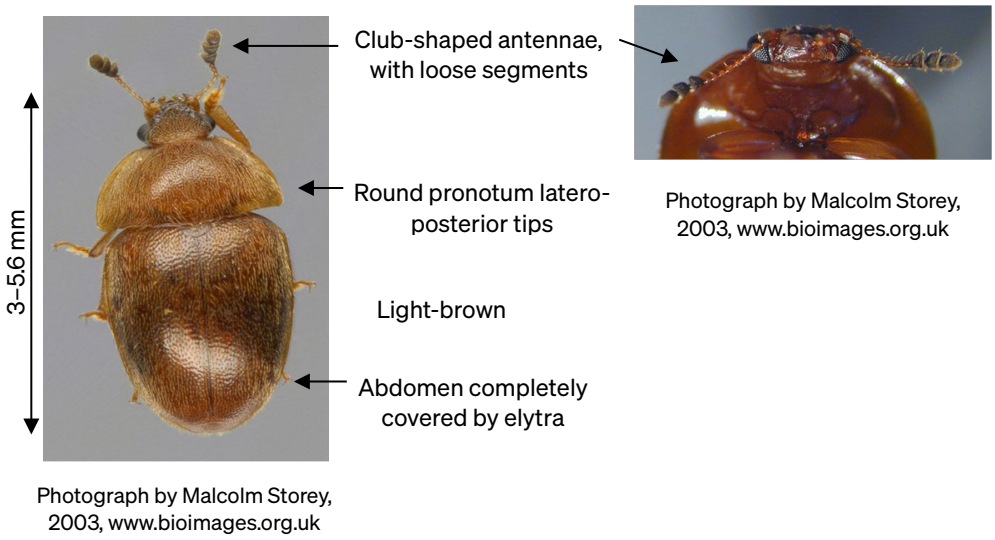


Fig. 10. **Cychramus luteus (*Neumann & Ritter, 2004*).**

Carpophilus lugubris has the following characteristics: (Figure 11; Marini *et al.*, 2013):

body is brown;
elytra have orange regions;
legs and antennae are orange (antennal clubs are dark orange);
body length: 3.3–4.5 mm.
However, as for *A. tumida:*
elytra do not cover the entire abdomen;
club-shaped antennae have compact segments;
latero-posterior tips of the pronotum are sharp.

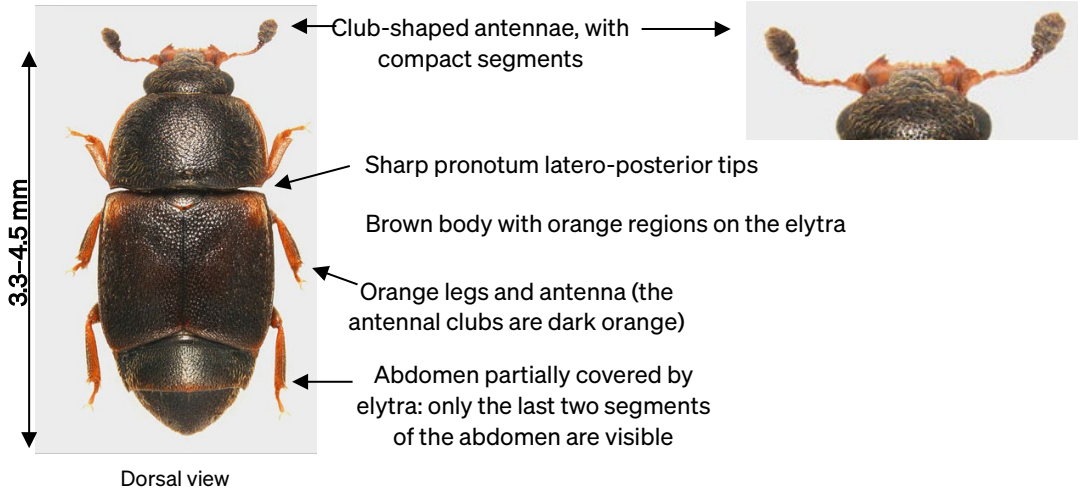


Fig. 11. **Carpophilus lugubris (*Marini* et al., *2013*).**

2.2.3.2. Larval form

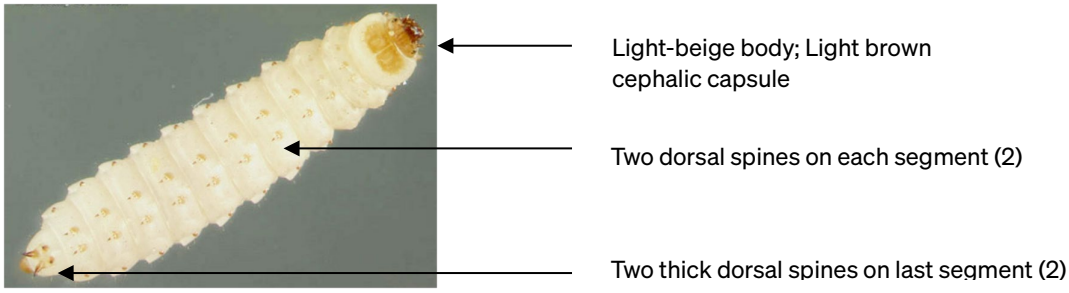
Larvae of *A. tumida* have a light beige body. The cephalic capsule (head of the larva) is brown. The colour may change with environmental conditions and conservation of the specimens. The body length at maturity is about 1 cm (1.2 cm maximum) depending on feeding. The width is about 1.6 mm.

Larva identification is based on the following morphological criteria: (see Figure 12).

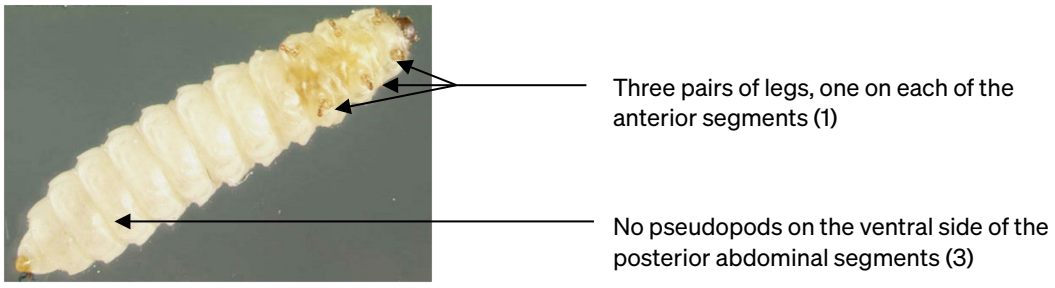
i) Three pairs of legs, one on each of the anterior (thoracic) segments

ii) Two dorsal spines on each segment (these spines are thicker on the last segment)

iii) No false legs (pseudopods or prolegs) on the ventral side of the posterior abdominal segments



Larva of *A. tumida*, dorsal view.



Larva of *A. tumida*, ventral view.

Fig. 12. Larva of* Aethina tumida*.
Photographs by Josephine Ratikan, University of Florida.

To distinguish *A. tumida* larvae from Lepidoptera larvae (lesser wax moth, *A. grisella* and honeycomb/greater wax moth, *G. mellonella*), frequently present in honeybee hives:

The Lepidoptera larvae have pseudopods on the ventral side of the abdominal segments.

There are two bare segments between the last segment with legs and the first segment with pseudopods (Figure 13).

The Lepidoptera larvae can make a silky web, cocoons, and have dark faeces (these webs and faeces may be observed in the sample containers received by the laboratory).

2.2.4. Interpretation of results

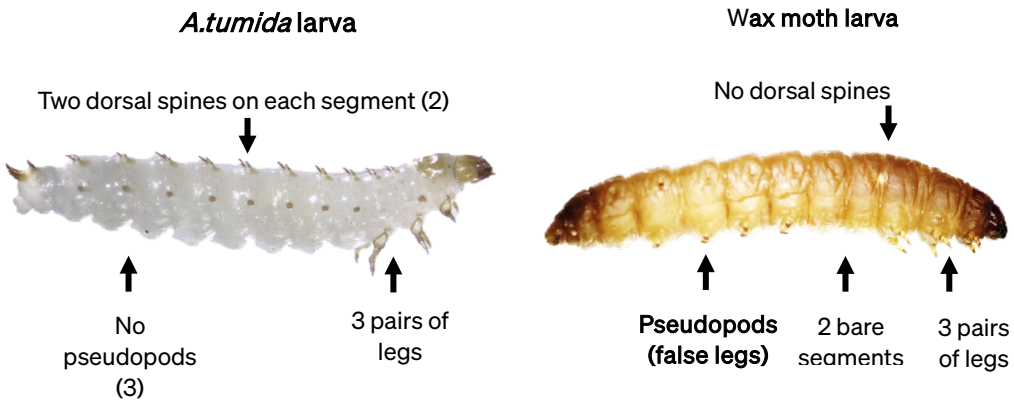


Fig. 13. Differentiation of A. tumida *from wax moth larvae. Photographs by Nicolas Cougoule. Anses, Sophia Antipolis laboratory.*

2.2.4.1. Adult form

i) If all the criteria 1 to 8 are confirmed for *A. tumida*, the result is "positive". The identification of *A. tumida* is confirmed. Confirmatory testing by PCR is advised.

ii) If certain fundamental morphological characteristics of *A. tumida* are not present (i.e. at least one out of the criteria 1 to 8), the result is "negative". The identification of *A. tumida* is not confirmed.

iii) Where definitive morphological criteria cannot be determined (e.g. damaged sample), the result is "inconclusive". Molecular identification is essential for confirmation.

2.2.4.2. Larval form

i) If all the criteria 1 to 3 are confirmed, the result is "*A. tumida* suspected". PCR testing is essential for final confirmation and confidence in the diagnosis.

ii) If at least one out of the criteria 1 to 3 is not confirmed, the result is "negative". The suspicion of *A. tumida* is not confirmed.

iii) Where definitive morphological criteria cannot be determined (e.g. damaged sample), the result is "inconclusive". Molecular identification is essential for confirmation.

2.3. Molecular identification

The morphological identification of the small hive beetle is increasingly confirmed by molecular methods using real-time PCR, especially for the examination of larvae where morphology is less clear cut. The method of real-time PCR described below has been developed by Ward *et al.* (2007) and is based on the amplification of a partial sequence of the mitochondrial gene of *A. tumida* that encodes cytochrome oxidase I (COI). The primers SHB207F and SHB315R can amplify a fragment of 109 base pairs, specific to *A. tumida*. This fragment is visualised in real-time due to a 5'-labelled probe. To take into account the two haplotypes identified by bioinformatic analysis by Ward *et al.* (2007), the SHB207F primer includes a degenerate base in the position 228 (A/G) (Genbank No. AF227645). The method was validated in accordance with Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*.

2.3.1. Sample preparation, equipment and reagents

The samples are typically adults or larvae kept in >90% non-denatured alcohol, or kept dry. Alcohol-preserved specimens should be left on tissue paper allowing the alcohol to evaporate or rinsed three times in a large volume of phosphate buffer (50 ml tube, for example). The specimen is then transferred to a 1.5 ml microtube where it is ground manually using a disposable pellet pestle. The volume depends on the size of the sample (for example: one adult beetle in 1 ml; one larva: in 200 µl). Samples can be stored at ≤ –16°C.

A real-time PCR detection system and the associated data analysis software are required to perform the test. Several proprietary systems for real-time PCR are available. The method described below uses one such system, but the precise parameters of the method should be validated according to the system in use in a particular laboratory. Because of the high sensitivity of the method, appropriate measures are required to avoid DNA contamination. All materials and methods used for the test should comply with the standards set out in the Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases*, including measures to prevent contamination of DNA in the specimen.

2.3.2. Preparation of reagents

The real-time PCR reaction mixture is usually provided as a ready to use 2× concentration. The manufacturer's instructions should be followed for use and storage. Working stock solutions for the primers and probe are prepared with nuclease-free TE buffer at the concentration of 20 µM and 50 µM respectively. The stock solutions are stored at –20°C and the probe should be protected from light. Single-use aliquots can be prepared to reduce the number of freeze–thawing cycles and to increase the shelf life of the primers and probes.

2.3.3. Real-time PCR test procedure

Primer/probe name	Sequence
SHB207F	5'-TCT-AAA-TAC-TAC-TTT-CTT-CGA-CCC-ATC-(A/G)-3'
SHB315R	5'-TCC-TGG-TAG-AAT-TAA-AAT-ATA-AAC-TTC-TGG-3'
SHB245T probe	5'-(6-FAM)-ATC-CAA-TCC-TAT-ACC-AAC-ACT-TAT-TTT-GAT-TCT-TCG-GAC-(TAMRA)-3'

Positive and negative extraction controls, as well as reagent controls, should be included in each PCR test. To minimise the risk of contamination by the positive control, a dilution resulting in a Ct value of about 30 should be used. A suitable control would be crushed *A. tumida* beetles diluted to 10 times the detection limit of the method (LD_{method}). Alternatively a plasmid containing the target sequence may be added diluted to 10 times the detection limit of the PCR (LD_{PCR}). For the negative extraction control, it is recommended to use the buffer used for crushing the specimens. An internal positive control (IPC) is highly recommended to check the absence of PCR inhibitor in the extract analysed.

Appropriate thermocycler conditions should be determined and validated for the equipment and reagents in use in the particular laboratory.

PCR reagent mixtures are added in a clean room (no pathogens or amplification products should be handled), for example:

	Final concentration	Volume for one tube (µl)
Nuclease free H_2O	/	4.1
Real-time PCR reaction mixture (2×)	1×	12.5
SHB207F (20 µM)	320 nM	0.4
SHB315R (20 µM)	320 nM	0.4
245 probe (50 µM)	100 nM	0.05
10× IPC Mix	1×	2.5
50× IPC DNA	0.1×	0.05
Mix total volume		20

Add 5 µl of the DNA template (unknown sample or plasmid DNA) or positive or negative control to the reagent mixture to a final volume of 25 µl. DNA samples are prepared and added to the PCR mix in a separate area.

The thermocycler programme will depend on the equipment used and the real-time PCR reaction mixture, for example:

Step	Cycle	Temperature (°C)	Time (minutes)
Polymerase activation	1	95	3:00
PCR	40	95	0:10
		60	0:30

2.3.4. Interpretation of results

The threshold for the analysis of the amplification curves (determined by the background noise associated with the detection system) is usually set according to the manufacturer's instructions for the software used. It can be performed on confirmed negative specimens (e.g. larvae of wax moth *Galleria melonella* or adult beetles of the genus *Meligethes*).

A result identifying *A. tumida* by real-time PCR is considered valid only if the positive extraction and PCR controls are positive (Ct $\leq$ 35) and if the negative extraction and PCR controls are negative (Ct = N/A).

A positive result is recorded for any sample with a Ct value <35. Negative results are for any sample with a Ct value >35 or which presents no Ct value. Samples giving negative results should be checked for the absence of PCR inhibitor in the extract analysed through the result of the IPC. PCR inhibitors can lead to false negative results. Inhibition may be overcome by dilution of the sample for example to 1/10.

3. Serological tests

Serological tests are not appropriate or relevant to bee colony infestations.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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FURTHER READING

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NB: There are WOAH Reference Laboratories for infestation with *Aethina tumida* (small hive beetle) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for infestation with *Aethina tumida* (small hive beetle)

NB: FIRST ADOPTED IN 2008. MOST RECENT UPDATES ADOPTED IN 2018.